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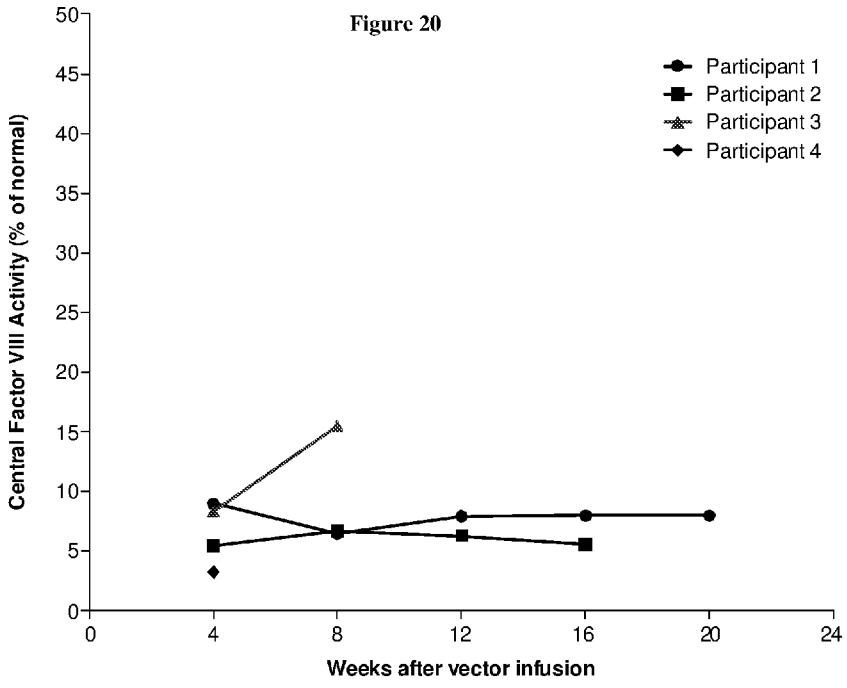
Figure 20



(57) Abstract: The invention provides expression cassettes. In certain embodiments, an expression cassette comprises (a) a regulatory element at least 90% identical to the sequence of any of SEQ ID NOs:2-67, and (b) a nucleic acid sequence encoding a Factor VIII protein having a B domain deletion (FVIII-BDD), where the nucleic acid sequence of (a) is at least 90% identical to the sequence of SEQ ID NO:77, where the regulatory element is operably linked to the nucleic acid sequence, and where no intron is present between the regulatory element and the nucleic acid sequence encoding FVIII-BDD, or where no more than 0 – 107 nucleotides of untranslated nucleic acid is between the regulatory element and the nucleic acid sequence encoding FVIII-BDD. In certain embodiments, expression cassettes contain sequence elements having CpG(s) substituted with CpT, CpA, TpG, or ApG at the same position(s) or has CpG reduced nucleic acid sequences.

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[Continued on next page]

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OPTIMIZED PROMOTER SEQUENCES, INTRON-FREE EXPRESSION CONSTRUCTS AND METHODS OF USE

Related Applications

[0001] This application claims priority to U.S. Provisional Patent Application No. 62/722,547, filed August 24, 2018, U.S. Provisional Patent Application No. 62/725,096, filed August 30, 2018, and U.S. Provisional Patent Application No. 62/784,116, filed December 21, 2018. The entire contents of the foregoing applications are incorporated herein by reference, including all text, tables, sequence listings and drawings.

Introduction

[0002] Gene therapy shows great promise in therapeutic applications involving loss of protein function or activity, for example, due to a genetic deficiency or defect, or the aberrant function or activity of a protein in which it is desired to suppress the expression of the aberrant protein. Improvements in transgene expression, enhancer and promoter function that drive transgene expression will enhance gene therapy therapeutic applications. The instant invention addresses, *inter alia*, this need.

Summary

[0003] In accordance with the instant invention expression cassettes comprising a nucleic acid sequence encoding a Factor VIII protein having a B domain deletion (FVIII-BDD) are provided.

[0004] In certain embodiments, the expression cassette comprises a sequence at least 98% identical to the sequence of SEQ ID NO:1, is at least 99% identical to the sequence of SEQ ID NO:1, comprises the sequence of SEQ ID NO:1, or consists of the sequence of SEQ ID NO:1.

[0005] In certain embodiments, the expression cassette comprises a regulatory element operably linked to a nucleic acid sequence encoding a Factor VIII protein having a B domain deletion (FVIII-BDD), wherein no intron is present between the regulatory element and the nucleic acid sequence, and wherein the expression cassette comprises a sequence at least 91% identical to the sequence of SEQ ID NO:1.

[0006] In certain embodiments, the expression cassette comprises (a) a regulatory element at least 90% identical to the sequence of any of SEQ ID NOs:2-67, and (b) a nucleic acid sequence encoding a Factor VIII protein having a B domain deletion (FVIII-BDD), wherein the nucleic acid sequence of (a) is at least 90% identical to the sequence of SEQ ID NO:77, and wherein the regulatory element is operably linked to the nucleic acid sequence, and wherein no intron is present between the regulatory element and the nucleic acid sequence encoding FVIII-BDD.

[0007] In certain embodiments, the expression cassette comprises (a) a regulatory element at least 90% identical to the sequence of any of SEQ ID NOs:2-67, and (b) a nucleic acid sequence encoding a Factor VIII protein having a B domain deletion (FVIII-BDD), wherein the nucleic acid sequence is at least 90% identical to the sequence of SEQ ID NO:77, and wherein the regulatory element is operably linked to the nucleic acid sequence, and wherein no more than 0 – 5, 5 – 10, 10 – 15, 15 – 20, 20 – 25, 25 – 30, 30 – 35, 35 – 40, 40 – 45, 45 – 50, 50 – 55, 55 – 60, 60 – 65, 65 – 70, 70 – 75, 75 – 80, 80 – 85, 85 – 90, 90 – 95, 95 – 100, 100 – 105, 106 or 107 nucleotides of untranslated nucleic acid is between the regulatory element and the nucleic acid sequence that encodes FVIII-BDD.

[0008] In certain embodiments, the regulatory element in the expression cassette comprises a nucleotide sequence at least 95% identical to any of SEQ ID NOs:2-67.

[0009] In certain embodiments, the regulatory element in the expression cassette has the same total number of reduced CpGs as set forth in the sequence of any of SEQ ID NOs:4-21 or 24-67.

[0010] In certain embodiments, the regulatory element in the expression cassette comprises the sequence of any of SEQ ID NOs:2-21 or 24-67 having CpG(s) substituted to be CpT, CpA, TpG, or ApG at the same position(s) as set forth in the sequence of any of SEQ ID NOs:4-21 or 24-67.

[0011] In certain embodiments, the nucleic acid sequence in the expression cassette exhibits greater expression when compared to expression from an expression cassette having (a) an intron, or (b) 108 or more nucleotides of untranslated nucleic acid, between the regulatory element and the nucleic acid sequence.

[0012] In certain embodiments, the encoded FVIII-BDD in the expression cassette exhibits greater biological activity as compared to expression from an expression cassette having (a) an intron, or (b) 108 or more nucleotides of untranslated nucleic acid, between the regulatory element and the nucleic acid sequence.

[0013] In certain embodiments, biological activity is determined by a clotting assay or reduced bleeding in a FVIII assay or FVIII deficiency model.

[0014] In certain embodiments, the expression cassette is more efficiently packaged into an AAV vector when compared to packaging of an expression cassette having (a) an intron, or (b) 108 or more nucleotides of untranslated nucleic acid, between the regulatory element and the nucleic acid sequence.

[0015] In accordance with the instant invention, cytosine-guanine dinucleotide (CpG) reduced nucleic acid sequences of regulatory elements (promoters) are provided. Exemplary promoters include the TTR (transthyretin gene) promoter and ApoE/hAAT (human apolipoprotein E gene /human alpha-1 antitrypsin gene) promoter. Exemplary promoters also include the fibrinogen gamma chain gene (FGG) promoter, the albumin promoter, and the serum amyloid A1 gene (SAA1) promoter. Exemplary promoters further include the TTR promoter fused to one or more of a hAAT promoter, FGG promoter, albumin promoter, and/or SAA1 promoter that result in a hybrid promoter or a promoter chimera.

[0016] CpG reduced nucleic acid regulatory elements include variants that exhibit altered gene expression levels compared to non-CpG reduced regulatory elements when transferred into cells. In certain embodiments, CpG reduced regulatory elements can provide for increased expression of a transgene or heterologous nucleic acid, such as a transgene encoding a protein such as a blood clotting factor (*e.g.*, FVIII), in a mammal, as well as provide increased efficacy in the context of gene transfer by increased circulating levels of the protein, such as a blood clotting factor, and achieving hemostasis for beneficial therapeutic outcomes.

[0017] In certain embodiments, a nucleic acid sequence has at least 1 fewer CpGs than a wild-type non-CpG reduced regulatory element (*e.g.*, any of SEQ ID NOs:2, 3, 22 and 23).

[0018] In certain embodiments, a nucleic acid sequence has at least 2 fewer CpGs than a wild-type non-CpG reduced regulatory element (*e.g.*, any of SEQ ID NOs:2, 3, 22 and 23).

[0019] In certain embodiments, a nucleic acid sequence has at least 3 fewer CpGs than a wild-type non-CpG reduced regulatory element (*e.g.*, any of SEQ ID NOs:2, 3, 22 and 23).

[0020] In certain embodiments, a nucleic acid sequence has at least 4 fewer CpGs than a wild-type non-CpG reduced regulatory element (*e.g.*, any of SEQ ID NOs:2, 3, 22 and 23).

[0021] In certain embodiments, a nucleic acid sequence has at least 5 fewer CpGs than a wild-type non-CpG reduced regulatory element (*e.g.*, any of SEQ ID NOs:22 and 23).

[0022] In certain embodiments, a nucleic acid sequence has at least 6 fewer CpGs than a wild-type non-CpG reduced regulatory element (*e.g.*, any of SEQ ID NOs:22 and 23).

[0023] In certain embodiments, a nucleic acid sequence has at least 7 fewer CpGs than a wild-type non-CpG reduced regulatory element (*e.g.*, any of SEQ ID NOs:22 and 23).

[0024] In certain embodiments, a nucleic acid sequence has at least 8 fewer CpGs than a wild-type non-CpG reduced regulatory element (*e.g.*, any of SEQ ID NOs:22 and 23).

[0025] In certain embodiments, a nucleic acid sequence has at least 9 fewer CpGs than a wild-type non-CpG reduced regulatory element (*e.g.*, any of SEQ ID NOs:22 and 23).

[0026] In certain embodiments, a nucleic acid sequence has at least 10 fewer CpGs than a wild-type non-CpG reduced regulatory element (*e.g.*, any of SEQ ID NOs:22 and 23).

[0027] In certain embodiments, a nucleic acid sequence has at least 11 fewer CpGs than a wild-type non-CpG reduced regulatory element (*e.g.*, any of SEQ ID NOs:22 and 23).

[0028] In certain embodiments, a nucleic acid sequence has at least 12 fewer CpGs than a wild-type non-CpG reduced regulatory element (*e.g.*, any of SEQ ID NOs:22 and 23).

[0029] In certain embodiments, a nucleic acid sequence has at least 13 fewer CpGs than a wild-type non-CpG reduced regulatory element (*e.g.*, any of SEQ ID NOs:22 and 23).

[0030] In certain embodiments, a nucleic acid sequence has at least 14 fewer CpGs than a wild-type non-CpG reduced regulatory element (*e.g.*, any of SEQ ID NOs:22 and 23).

[0031] In certain embodiments, a nucleic acid sequence has at least 15 fewer CpGs than a wild-type non-CpG reduced regulatory element (*e.g.*, any of SEQ ID NOs:22 and 23).

[0032] In certain embodiments, a nucleic acid sequence has at least 16 fewer CpGs than a wild-type non-CpG reduced regulatory element (*e.g.*, any of SEQ ID NOs:22 and 23).

[0033] In certain embodiments, a nucleic acid sequence has no more than 16 CpGs; has no more than 15 CpGs; has no more than 14 CpGs; has no more than 13 CpGs; has no more than 12 CpG; has no more than 11 CpGs; has no more than 10 CpGs; has no more than 9 CpGs; has no more than 8 CpGs; has no more than 7 CpGs; has no more than 6 CpGs; has no more than 5 CpGs; has no more than 4 CpGs; has no more than 3 CpGs; has no more than 2 CpGs; or has no more than 1 CpG.

[0034] In certain embodiments, a nucleic acid sequence has at most15 CpGs; 14 CpGs; 13 CpGs; 12 CpGs; 11 CpG; 10 CpGs; 9 CpGs; 8 CpGs; 7 CpGs; 6 CpGs; 5 CpGs; 4 CpGs; 3 CpGs; 2 CpGs; or 1 CpG. In certain embodiments, a nucleic acid sequence has no CpGs.

[0035] In certain embodiments, a nucleic acid sequence comprising SEQ ID NO:22 or 23 is modified to have 15 or fewer cytosine-guanine dinucleotides (CpGs); 14 or fewer CpGs; 13 or fewer CpGs; 12 or fewer CpGs; 11 or fewer CpGs; 10 or fewer CpGs; 9 or fewer CpGs; 8 or fewer CpGs; 7 or fewer CpGs; 6 or fewer CpGs; 5 or fewer CpGs; 4 or fewer CpGs; 3 or fewer CpGs; 2 or fewer CpGs; or 1 or 0 CpGs. In certain embodiments, the nucleic acid sequence in any of SEQ ID NOs:2, 3, 22 and 23 is modified to have 0 CpG.

[0036] In certain embodiments, the nucleic acid sequence in any of SEQ ID NOs:2, 3, 22 and 23 is modified such that the C in every CpG site is modified to a T, optionally excluding the C in the C/EBP site.

[0037] In certain embodiments, the nucleic acid sequence in any of SEQ ID NOs:2, 3, 22 and 23 is modified such that the C in every CpG site is modified to a T, optionally excluding the C in the C/EBP site, except that the first CpG site is left unmodified.

[0038] In certain embodiments, the nucleic acid sequence in any of SEQ ID NOs:2, 3, 22 and 23 is modified such that the C in every CpG site is modified to a T, optionally excluding the C in the C/EBP site, except that the second CpG site is left unmodified.

[0039] In certain embodiments, the nucleic acid sequence in any of SEQ ID NOs:2, 3, 22 and 23 is modified such that the C in every CpG site is modified to a T, optionally excluding the C in the C/EBP site, except that the third CpG site is left unmodified.

[0040] In certain embodiments, the nucleic acid sequence in any of SEQ ID NOs:2, 3, 22 and 23 is modified such that the C in every CpG site is modified to a T, optionally excluding the C in the C/EBP site, except that the fourth CpG site is left unmodified.

[0041] In certain embodiments, the nucleic acid sequence in any of SEQ ID NOs:22 and 23 is modified such that the C in every CpG site is modified to a T, optionally excluding the C in the C/EBP site, except that the fifth CpG site is left unmodified.

[0042] In certain embodiments, the nucleic acid sequence in any of SEQ ID NOs:22 and 23 is modified such that the C in every CpG site is modified to a T, optionally excluding the C in the C/EBP site, except that the sixth CpG site is left unmodified.

[0043] In certain embodiments, the nucleic acid sequence in any of SEQ ID NOs:22 and 23 is modified such that the C in every CpG site is modified to a T, except that the seventh CpG site is left unmodified.

[0044] In certain embodiments, the nucleic acid sequence in any of SEQ ID NOs:22 and 23 is modified such that the C in every CpG site is modified to a T, optionally excluding the C in the C/EBP site, except that the eighth CpG site is left unmodified.

[0045] In certain embodiments, the nucleic acid sequence in any of SEQ ID NOs:22 and 23 is modified such that the C in every CpG site is modified to a T, optionally excluding the C in the C/EBP site, except that the ninth CpG site is left unmodified.

[0046] In certain embodiments, the nucleic acid sequence in any of SEQ ID NOs:22 and 23 is modified such that the C in every CpG site is modified to a T, optionally excluding the C in the C/EBP site, except that the tenth CpG site is left unmodified.

[0047] In certain embodiments, the nucleic acid sequence in any of SEQ ID NOs:22 and 23 is modified such that the C in every CpG site is modified to a T, optionally excluding the C in the C/EBP site, except that the eleventh CpG site is left unmodified.

[0048] In certain embodiments, the nucleic acid sequence in any of SEQ ID NOs:22 and 23 is modified such that the C in every CpG site is modified to a T, optionally excluding the C in the C/EBP site, except that the twelfth CpG site is left unmodified.

[0049] In certain embodiments, the nucleic acid sequence in any of SEQ ID NOs:22 and 23 is modified such that the C in every CpG site is modified to a T, optionally excluding the C in the C/EBP site, except that the thirteenth CpG site is left unmodified.

[0050] In certain embodiments, the nucleic acid sequence in any of SEQ ID NOs:22 and 23 is modified such that the C in every CpG site is modified to a T, optionally excluding the C in the C/EBP site, except that the fourteenth CpG site is left unmodified.

[0051] In certain embodiments, the nucleic acid sequence in any of SEQ ID NOs:22 and 23 is modified such that the C in every CpG site is modified to a T, optionally excluding the C in the C/EBP site, except that the fifteenth CpG site is left unmodified.

[0052] In certain embodiments, the nucleic acid sequence in any of SEQ ID NOs:22 and 23 is modified such that the C in every CpG site is modified to a T, optionally excluding the C in the C/EBP site, except that the sixteenth CpG site is left unmodified.

[0053] In certain embodiments, the nucleic acid sequence is modified such that at least the 1st CpG from the 5' end in any of SEQ ID NOs:2, 3, 22 and 23 is modified to not be CpG.

[0054] In certain embodiments, the nucleic acid sequence is modified such that at least the 2nd CpG from the 5' end in in any of SEQ ID NOs:2, 3, 22 and 23 is modified to not be CpG.

[0055] In certain embodiments, the nucleic acid sequence is modified such that at least the 3rd CpG from the 5' end in in any of SEQ ID NOs:2, 3, 22 and 23 is modified to not be CpG.

[0056] In certain embodiments, the nucleic acid sequence is modified such that at least the 4th CpG from the 5' end in in any of SEQ ID NOs:2, 3, 22 and 23 is modified to not be CpG.

[0057] In certain embodiments, the nucleic acid sequence is modified such that at least the 5th CpG from the 5' end in in any of SEQ ID NOs:22 and 23 is modified to not be CpG.

[0058] In certain embodiments, the nucleic acid sequence is modified such that at least the 6th CpG from the 5' end in in any of SEQ ID NOs:22 and 23 is modified to not be CpG.

[0059] In certain embodiments, the nucleic acid sequence is modified such that at least the 7th CpG from the 5' end in in any of SEQ ID NOs:22 and 23 is modified to not be CpG.

[0060] In certain embodiments, the nucleic acid sequence is modified such that at least the 8th CpG from the 5' end in in any of SEQ ID NOs:22 and 23 is modified to not be CpG.

[0061] In certain embodiments, the nucleic acid sequence is modified such that at least the 9th CpG from the 5' end in in any of SEQ ID NOs:22 and 23 is modified to not be CpG.

[0062] In certain embodiments, the nucleic acid sequence is modified such that at least the 10th CpG from the 5' end in in any of SEQ ID NOs:22 and 23 is modified to not be CpG.

[0063] In certain embodiments, the nucleic acid sequence is modified such that at least the 11th CpG from the 5' end in in any of SEQ ID NOs:22 and 23 is modified to not be CpG.

[0064] In certain embodiments, the nucleic acid sequence is modified such that at least the 12th CpG from the 5' end in in any of SEQ ID NOs:22 and 23 is modified to not be CpG.

[0065] In certain embodiments, the nucleic acid sequence is modified such that at least the 13th CpG from the 5' end in in any of SEQ ID NOs:22 and 23 is modified to not be CpG.

[0066] In certain embodiments, the nucleic acid sequence is modified such that at least the 14th CpG from the 5' end in in any of SEQ ID NOs:22 and 23 is modified to not be CpG.

[0067] In certain embodiments, the nucleic acid sequence is modified such that at least the 15th CpG from the 5' end in in any of SEQ ID NOs:22 and 23 is modified to not be CpG.

[0068] In certain embodiments, the nucleic acid sequence is modified such that at least the 16th CpG from the 5' end in in any of SEQ ID NOs:22 and 23 is modified to not be CpG.

[0069] In certain embodiments, the cytosine of one or more CpGs in any of SEQ ID NOs:2, 3, 22 and 23 is modified to a thymine. In certain embodiments, the cytosine of one or more CpGs in any of SEQ ID NOs:2, 3, 22 and 23 is modified to adenine.

[0070] In certain embodiments, the nucleic acid sequence in any of SEQ ID NOs:2, 3, 22 and 23 is modified such that the C in one or more CpGs is deleted.

[0071] In certain embodiments, the nucleic acid sequence in any of SEQ ID NOs:2, 3, 22 and 23 is modified such that the G in one or more CpGs is deleted.

[0072] In certain embodiments, the nucleic acid sequence in any of SEQ ID NOs:2, 3, 22 and 23 is modified such that the C and G in one or more CpGs are deleted.

[0073] Exemplary CpG reduced TTR promoters are set forth in SEQ ID NOs:4 – 13.

[0074] Exemplary CpG reduced hybrid promoters are set forth in SEQ ID NOs:14 – 21.

[0075] Exemplary CpG reduced ApoE/hAAT promoters are set forth in SEQ ID NOs:24 – 67.

[0076] In certain embodiments, the cytosine of one or more CpGs in a hybrid promoter, comprising all or a portion of the TTR promoter fused to all or a portion of at least one of the hAAT promoter, and/or the FGG promoter, and/or the albumin promoter, and/or the SAA1 promoter, is modified to a thymine (C→T) or an adenine (G→A). The TTR promoter can be fused to any one or a combination of the foregoing promoters in any 5' → 3' orientation.

[0077] In certain embodiments, a hybrid promoter has a 5' → 3' orientation, TTR/hAAT or hAAT/TTR. In certain embodiments, a hybrid promoter has a 5' → 3' orientation, TTR/FGG or FGG/TTR. In certain embodiments, a hybrid promoter has a 5' → 3' orientation, TTR/hAAT/albumin or hAAT/TTR/albumin or albumin/TTR/hAAT or TTR/albumin/hAAT, hAAT/albumin/TTR or albumin/hAAT/TTR, etc.

[0078] In certain embodiments, a hybrid promoter has a 5' → 3' orientation, TTR/FGG/albumin or hAAT/TTR/FGG or FGG/TTR/hAAT or TTR/FGG/hAAT, etc.

[0079] In certain embodiments, a hybrid promoter has a TTR promoter fused to all or a portion of all 4 of the hAAT promoter, the FGG promoter, the albumin promoter, and the SAA1 promoter. The TTR promoter can be fused to all or a portion of the foregoing promoters in any 5' → 3' orientation and in any promoter order.

[0080] In certain embodiments, a nucleic acid sequence or polynucleotide of the instant invention, such as a CPG reduced nucleic acid sequence, is operably linked to a transgene.

[0081] In certain embodiments, a nucleic acid sequence or polynucleotide of the instant invention, such as modified SEQ ID NO:2, 3, 22 or 23 as set forth herein, is operably linked to transgene.

[0082] In certain embodiments, a nucleic acid sequence or polynucleotide of the instant invention, such as modified SEQ ID NO:2, 3, 22 or 23 as set forth herein, confers transcription on an operably linked transgene that is within about 5-100% of the transcription conferred by unmodified SEQ ID NO:2, 3, 22 or 23, or that is within about 50% of the transcription conferred by unmodified SEQ ID NO:2, 3, 22 or 23 , or that is within about 25-50% of the transcription conferred by unmodified SEQ ID NO:2, 3, 22 or 2.

[0083] In certain embodiments, a nucleic acid sequence or polynucleotide, such as modified SEQ ID NO:2, 3, 22 or 23 as set forth herein, is positioned 5' of a transgene.

[0084] In certain embodiments, a transgene encodes a blood coagulation or clotting protein.

[0085] In certain embodiments, a transgene encodes Factor IX (FIX), Factor VIII (FVIII), Factor VII (FVII) or Protein C.

[0086] In certain embodiments, a transgene encodes Factor VIII having a sequence at least 95% identical to the sequence of SEQ ID NO:68.

[0087] In certain embodiments, a transgene is transcribed into an inhibitory RNA. In certain embodiments, an inhibitory RNA comprises antisense RNA, a microRNA (miRNA), or a small interfering RNA (siRNA).

[0088] In certain embodiments, a transgene encodes a therapeutic protein that is expressed in liver cells and secreted into the systemic circulation.

[0089] In certain embodiments, the therapeutic protein treats or prevents a neurodegenerative or central nervous system (CNS) disease.

[0090] In certain embodiments, the therapeutic protein is a protective ApoE isoform.

[0091] In certain embodiments, the therapeutic protein is ApoE ε2 isoform.

[0092] In certain embodiments, the therapeutic protein treats or prevents an autoimmune disease or allergic disease.

[0093] In certain embodiments, the therapeutic protein is a fusion protein comprising an unwanted antigen and a leader sequence that drives secretion of said therapeutic protein from the cell.

[0094] In certain embodiments, the unwanted antigen is the extracellular domain of myelin oligodendrocyte glycoprotein (MOG) or a fragment thereof.

[0095] In certain embodiments, an expression cassette comprises a nucleic acid sequence or polynucleotide of the instant invention, such as a modified SEQ ID NO:2, 3, 22 or 23 as set forth herein, operably linked to a transgene, in which the nucleic acid sequence or polynucleotide is positioned upstream of the 5' end of the transgene and optionally wherein there are no more than 0 – 5, 5 – 10, 10 – 15, 15 – 20, 20 – 25, 25 – 30, 30 – 35, 35 – 40, 40 – 45, 45 – 50, 50 – 55, 55 – 60, 60 – 65, 65 – 70, 70 – 75, 75 – 80, 80 – 85, 85 – 90, 90 – 95, 95 – 100, 100 – 105, 106 or 107 nucleotides of untranslated nucleic acid sequence positioned between the nucleic acid sequence or polynucleotide and the 5' end of the transgene.

[0096] In certain embodiments, an expression cassette comprises a first nucleotide sequence having 95% or greater sequence identity to the sequence of any of SEQ ID NOs:4-21 or 24-67, in which the first nucleotide sequence positioned upstream of the 5' end of a second nucleotide sequence has 95% or greater sequence identity to the sequence of SEQ ID NO:77, and optionally wherein there are no more than 0 – 5, 5 – 10, 10 – 15, 15 – 20, 20 – 25, 25 – 30, 30 – 35, 35 – 40, 40 – 45, 45 – 50, 50 – 55, 55 – 60, 60 – 65, 65 – 70, 70 – 75, 75 – 80, 80 – 85, 85 – 90, 90 – 95, 95 – 100, 100 – 105, 106 or 107 nucleotides of untranslated nucleic acid sequence positioned between the first nucleotide sequence and the 5' end of the second nucleotide sequence.

[0097] In certain embodiments, the expression cassette comprises the sequence of SEQ ID NO:1 or a polynucleotide having at least 98% sequence identity to the sequence of SEQ ID NO:1.

[0098] In certain embodiments, the expression cassette comprises a polynucleotide having at least 99% sequence identity to the sequence of SEQ ID NO:1.

[0099] In certain embodiments, the expression cassette consists essentially of SEQ ID NO:1.

[0100] In certain embodiments, the transgene or second nucleotide sequence comprises a nucleic acid sequence encoding Factor VIII (FVIII) having a B-domain deletion (FVIII-BDD), and the nucleic acid sequence encodes a FVII-BDD protein with FVIII blood coagulation activity and having at least 90% sequence identity to the sequence of SEQ ID NO:68.

[0101] In certain embodiments, the transgene or second nucleotide sequence comprises a nucleic acid sequence encoding Factor VIII (FVIII) having a B-domain deletion (FVIII-BDD), and the nucleic acid sequence has 90% or greater sequence identity to the sequence of SEQ ID NO:77 and encodes a protein having FVIII blood coagulation activity.

[0102] In certain embodiments, the untranslated nucleic acid sequence is not an intron or is intron-free.

[0103] In certain embodiments, the first nucleotide sequence comprises a nucleic acid sequence at least 95% identical to any of SEQ ID NOs:4-21 or 24-67.

[0104] In certain embodiments, the first nucleotide sequence comprises a nucleic acid sequence at least 95% identical to the sequence of any of SEQ ID NOs:4-21 or 24-67, and has the same total number of reduced CpGs as set forth in the sequence of any of SEQ ID NOs:4-21 or 24-67.

[0105] In certain embodiments, the first nucleotide sequence comprises a nucleic acid sequence at least 95% identical to the sequence of any of SEQ ID NOs:4-21 or 24-67, and having CpG(s) substituted to be CpT, CpA, TpG, or ApG at the same position(s) as set forth in the sequence of any of SEQ ID NOs:4-21 or 24-67.

[0106] In certain embodiments, the second nucleotide sequence exhibits greater expression when compared to expression from a polynucleotide having 108 or more nucleotides between the first nucleotide sequence and the 5' end of the second nucleotide sequence.

[0107] In certain embodiments, the second nucleotide sequence exhibits greater biological activity when compared to expression from a polynucleotide having 108 or more nucleotides between the first nucleotide sequence and the 5' end of the second nucleotide sequence.

[0108] In certain embodiments, biological activity is determined by a clotting assay or reduced bleeding in a FVIII assay or FVIII deficiency model.

[0109] In certain embodiments, the second nucleotide sequence is more efficiently packaged into an AAV vector when compared to packaging of a polynucleotide having 108 or more nucleotides between the first nucleotide sequence and the 5' end of the second nucleotide sequence.

[0110] In certain embodiments, an adeno-associated virus (AAV) vector comprises the nucleic acid sequence, or polynucleotide or expression cassette as set forth herein.

[0111] In certain embodiments, the AAV vector comprises one or more of: a) an AAV capsid;-and b) one or more AAV inverted terminal repeats (ITRs), wherein the AAV ITR(s) flanks the 5' or 3' terminus of the nucleic acid sequence, the polynucleotide, and/or the transgene.

[0112] In certain embodiments, the AAV vector further comprises an intron positioned within the flanking 5' or 3' ITR.

[0113] In certain embodiments, the intron or one or more ITRs is modified to have reduced CpGs.

[0114] In certain embodiments, the AAV capsid serotype comprises a modified or variant AAV VP1, VP2 and/or VP3 capsid having 90% or more sequence identity to AAV1, AAV2, AAV3, AAV3B, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, Rh10, Rh74, AAV-2i8, SEQ ID NO:91 or SEQ ID NO:92 VP1, VP2 and/or VP3 sequences, or a capsid having 95% or more sequence identity to AAV1, AAV2, AAV3, AAV3B, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, Rh10, Rh74, AAV-2i8, SEQ ID NO:91 or SEQ ID NO:92 VP1, VP2 and/or VP3 sequences, or a capsid having 100% sequence identity to AAV1, AAV2, AAV3, AAV3B, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, Rh10, Rh74, AAV-2i8, SEQ ID NO:91 or SEQ ID NO:92 VP1, VP2 and/or VP3 sequences.

[0115] In certain embodiments, the ITRs comprise one or more ITRs of any of: AAV1, AAV2, AAV3, AAV3B, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, Rh10, or Rh74 AAV serotypes, or a combination thereof.

[0116] In certain embodiments, the AAV vector further comprises an ITR, a polyA signal and/or intron sequence.

[0117] In certain embodiments, the AAV vectors as set forth herein are any pharmaceutical composition.

[0118] In certain embodiments, a pharmaceutical composition comprises a biologically compatible carrier or excipient.

[0119] In certain embodiments, a pharmaceutical composition further comprises empty AAV capsids.

[0120] In certain embodiments, a composition or a pharmaceutical composition comprises a ratio of empty AAV capsids to AAV vectors within or between about 100:1-50:1, from about 50:1-25:1, from about 25:1-10:1, from about 10:1-1:1, from about 1:1-1:10, from about 1:10-1:25, from about 1:25-1:50, or from about 1:50-1:100.

[0121] In certain embodiments, the ratio of the empty AAV capsids to the AAV vectors in a composition or pharmaceutical composition is about 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, or 10:1

[0122] In certain embodiments, a composition or a pharmaceutical composition set forth herein further comprises a surfactant.

[0123] In certain embodiments, methods of treating a human in need of gene therapy are provided.

[0124] In certain embodiments, the human is in need of a blood coagulation or clotting factor.

[0125] In certain embodiments, a method of treating a human includes (a) providing an expression cassette as set forth herein, a polynucleotide as set forth herein, an AAV vector as set forth herein, or a pharmaceutical composition as set forth herein; and (b) administering an amount of the expression cassette, polynucleotide, AAV vector, or pharmaceutical composition to the human, wherein the blood coagulation or clotting factor is expressed in the human.

[0126] In certain embodiments, the human has hemophilia A or B.

[0127] In certain embodiments, the AAV vector is administered to the human intravenously, intraarterially, intra-cavity, intramucosally, or via catheter.

[0128] In certain embodiments, the blood coagulation or clotting factor is expressed at increased levels after administration.

[0129] In certain embodiments, the blood coagulation or clotting factor is expressed at greater than 1% of the levels of the blood coagulation or clotting factor found in a human not in need of blood coagulation or clotting factor.

[0130] In certain embodiments, the blood coagulation or clotting factor is expressed at about 1%-40% of the levels of the blood coagulation or clotting factor found in a human not in need of blood coagulation or clotting factor.

[0131] In certain embodiments, the blood coagulation or clotting factor is expressed at about 5%-30% of the levels of the blood coagulation or clotting factor found in a human not in need of blood coagulation or clotting factor.

[0132] In certain embodiments, the AAV vector is administered in a range from about $1X10^{8}$ to about $1X10^{14}$ vector genomes per kilogram (vg/kg) of the weight of the human.

Description of Drawings

[0133] **Figure 1** shows human Factor IX (hFIX) levels, measured by an activity assay, in the plasma of mice 24 hours post hydrodynamic delivery of CpG reduced ApoE/hAAT regulatory element-hFIX encoding constructs, labeled "CpG1" through "CpG22". SEQ ID NOs:24-67 correspond to regulatory elements CpG1-ApoE/hAAT through CpG22-ApoE/hAAT, respectively, with and without restriction enzyme sites, and are further described in Example 13. Levels hFIX are presented as fold of a reference plasmid containing non-CpG reduced ApoE/hAAT (SEQ ID NO:23).

[0134] **Figure 2** shows human Factor VIII (hFVIII) antigen levels, measured by an ELISA assay, in the plasma of mice 24 hours post hydrodynamic delivery of CpG reduced TTRm promoter hFVIII-encoding constructs, labeled "CpG1" through "CpG5". SEQ ID NOs:4-21 correspond to the promoters CpG1-TTRm through CpG5-TTRm, respectively, with and

without flanking restriction enzyme sites, and are further described in Example 13. Levels are presented as percentage of normal human plasma FVIII, where 100% = 150 ng/mL. "TTRm" refers to a hFVIII-encoding construct containing a non-CpG reduced TTRm promoter (SEQ ID NO:3).

[0135] **Figure 3** shows hFVIII antigen levels, measured by an ELISA assay, in plasma of mice 24 hours post hydrodynamic delivery of CpG reduced hybrid promoter hFVIII-encoding constructs, labeled "Hybrid 6" through "Hybrid 9." SEQ ID NOs:14-21 correspond to CpG reduced promoters Hybrid6 through Hybrid9, respectively. Levels are presented as percentage of normal human plasma FVIII, where 100% = 150 ng/mL. "TTRm" refers to hFVIII-encoding construct containing a non-CpG reduced TTRm promoter (SEQ ID NO:3).

[0136] **Figure 4** shows hFVIII antigen levels, measured by an ELISA assay, in the plasma of mice 2, 4 and 8 weeks post-delivery of AAV encapsidated non-CpG reduced (TTRm) and CpG reduced Hybrid 6, 7, 8 and 9 promoter-hFVIII constructs, at a dose of 6.4e11 vector genomes (vg)/kg. Levels are presented as percentage of normal human plasma FVIII, where 100% = 150 ng/mL.

[0137] **Figure 5** shows hFVIII antigen levels measured by an ELISA assay, in the plasma of mice 8 weeks post-delivery of AAV encapsidated non-CpG reduced (TTRm-hFVIII), Hybrid 7 and Hybrid 9 promoter-hFVIII constructs, at a dose range of 2.56e11, 6.4e11 and 1.6e12 vg/kg. Levels are presented as percentage of normal human plasma FVIII, where 100% = 150 ng/mL.

[0138] **Figure 6** shows expression of hFVIII mRNA in liver, brain, testes, spleen and kidney of mice 8 weeks following intravenous administration of AAV encapsidated non-CpG reduced (TTRm-hFVIII), Hybrid 7 and Hybrid 9 promoter-hFVIII constructs at a dose of 6.4e11 vg/kg. No hFVIII RNA was observed in any tissues other than the liver, demonstrating the liver-specific nature of the promoters. Groups of results for liver, brain, testes, spleen and kidney are shown from left to right for TTRm-hFVIII, Hybrid7-hFVIII, and Hybrid9-hFVIII, respectively.

[0139] **Figure 7** shows a schematic comparison of an expression cassette with an intron, referred to as "AAV-WINT" (TTRm-intron-hFVIII-BDD) and an expression cassette without an intron, referred to as "AAV-INTL" (TTRm-hFVIII-BDD intronless; SEQ ID NO:1).

AAV-WINT has a synthetic intron (SEQ ID NO:93) located between a TTRm promoter and a transgene encoding B-domain deleted human Factor VIII(hFVIII-BDD), which is not present in AAV-INTL. The codon-optimized nucleic acid sequence in these cassettes encoding hFVIII-BDD is set forth in SEQ ID NO:77.

[0140] **Figure 8** shows hFVIII levels as detected by ELISA performed on mouse plasma samples for the 6.4e9 vg/mouse dosage from study #1. Results are the average of animals in each treatment group (n=5). Error bars represent standard deviation.

[0141] **Figure 9** shows hFVIII levels as detected by ELISA performed on mouse plasma samples for the 1.6e10 vg/mouse dosage from study #1. Results are the average of animals in each treatment group (TTRm hFVIII, n=4; TTRm hFVIII intronless, n=5). Error bars represent standard deviation.

[0142] **Figure 10** shows hFVIII levels as detected by ELISA performed on mouse plasma samples from study #2. Results are the average of animals in each treatment group (n=10). Error bars represent standard deviation.

[0143] **Figure 11** shows hFVIII levels as detected by ELISA performed on non-human primate (NHP) plasma samples from study #1. Results from individual monkeys in low dose (2e12 vg/kg) group 1 (AAV-WINT, lines with boxes, n=2) and group 2 (AAV-INTL, SEQ ID NO:1, lines with triangles, n=3) are shown. One animal, P0001, from low dose AAV-WINT was removed due to positive neutralizing antibodies to AAV observed in pre-dose day -8 sample.

[0144] **Figure 12** shows hFVIII levels as detected by ELISA performed on NHP plasma samples from study #1. Results from individual monkeys in high dose (6e12 vg/kg) group 3 (AAV-WINT, lines with boxes, n=2) and group 4 (AAV-INTL, SEQ ID NO:1, lines with triangles, n=3) are shown. One animal, P0101, from high dose AAV-WINT was removed due to no observed hFVIII expression upon treatment.

[0145] **Figure 13** shows hFVIII levels as detected by ELISA performed on plasma samples of NHPs from study #2. Results from individual monkeys at the 2e12 vg/kg dose for AAV-WINT (lines with boxes, n=5) and AAV-INTL, (SEQ ID NO:1, lines with triangles, n=5) are shown.

[0146] **Figure 14** shows the results of a cell-based vector potency assay at three different multiplicities of infection (MOI). Cell supernatants were assessed for hFVIII activity by Chromogenix Coatest SP4 and are the average of two biological replicates assayed in duplicate. Error bars represent standard deviation. "AAV-WINT" and "AAV-INTL" are original, undiluted stock vials of virus, whereas "AAV-WINT Dosing" and "AAV-INTL Dosing" indicate materials diluted for infusion.

[0147] **Figure 15** shows results from Figure 14 replotted as fold-change versus AAV-WINT for each MOI.

[0148] **Figure 16** shows an evaluation of vector potency in an *in vitro*, cell-based vector potency assay at three different MOI. Cell supernatants were assessed for hFVIII activity by Chromogenix Coatest SP4 and are the average of two biological replicates assayed in duplicate. Error bars represent standard deviation. AAV-WINT and AAV-INTL (SEQ ID NO:1) are original stock vials of virus from two different lots.

[0149] **Figure 17** shows a comparison of expression cassettes for *in vitro* hFVIII levels, assayed by Chromogenix Coatest SP4 from supernatants of Huh7 cells transfected with independent plasmid DNA preparations (prep) of mTTR-intron-hFVIII-BDD and mTTR-hFVIII-BDD (SEQ ID NO:1). Individual data points are shown as filled circles, with each bar representing the average of two biological replicates (n=2) assayed in duplicate. Error bars represent standard deviation.

[0150] **Figure 18** shows daily FVIII activity levels in 4 human subjects (participant 1 (circle), 2 (square), 3 (triangle) and 4 (diamond)) infused with $5x10^{11}$ vg/kg of AAV-INTL hFVIII expression cassette (SEQ ID NO:1) encapsidated in LK03 AAV vector (SEQ ID NO:91), referred to herein as LK03-INTL hFVIII-BDD.

[0151] **Figure 19** shows weekly averages of FVIII activity levels in 4 human subjects (participant 1 (circle), 2 (square), 3 (triangle) and 4 (diamond)) infused with $5x10^{11}$ vg/kg of LK03-INTL hFVIII-BDD.

[0152] **Figure 20** shows four-week block averages of FVIII activity levels in 4 human subjects (participant 1 (circle), 2 (square), 3 (triangle) and 4 (diamond)) infused with $5x10^{11}$ vg/kg of LK03-INTL hFVIII-BDD.

Detailed Description

[0153] Disclosed herein are intron-free expression cassettes for the expression of Factor VIII (FVIII) and FVIII having a deleted B-domain (FVIII-BBD) (*e.g.*, human FVIII (hFVIII) and human FVIII-BDD (hFVIII-BDD)). Investigators have reported that inclusion of an intron in an expression cassette, including in AAV delivery vectors, can contribute to increased transgene expression (Huang *et al.*, 1990, Nucl. Acid Res., 18:937-947; Choi *et al.*, 2014, Mol. Brain, 7:17; Powell *et al.*, 2015, Discov. Med., 19:49-57; Lu *et al.*, 2017, Hum. Gene Ther., 28:125-134). Surprisingly, as disclosed herein, partial or complete removal of an intron led to increased AAV vector potency and transgene (in this case Factor VIII) expression levels in cell culture, mice and non-human primates. The "intron-free" expression cassette design is an improvement in vectors for treatment of blood clotting disorders such as Hemophilia A, and may provide efficacy at lower vector doses, potentially offering benefits to patient safety and outcomes, in addition to decreasing barriers to manufacturing, such as costs and time.

[0154] Also disclosed herein are nucleic acid sequences having reduced CpGs compared with a reference wild-type mammalian (*e.g.*, human) sequence and/or less than 100% sequence identity with a reference wild-type mammalian (*e.g.*, human) sequence. Nucleic acid sequences having reduced CpGs include one or more the following promoters: TTR promoter, ApoE/hAAT promoter, FGG promoter, albumin promoter, and SAA1 promoter. Nucleic acid sequences having reduced CPGs include fusions or hybrids of TTR promoter and at least one or more the following promoters: ApoE/hAAT promoter, FGG promoter, albumin promoter, and SAA1 promoter.

[0155] The terms "polynucleotide" and "nucleic acid" are used interchangeably herein to refer to all forms of nucleic acid, oligonucleotides, including deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Polynucleotides include genomic DNA, cDNA and antisense DNA, and spliced or unspliced mRNA, rRNA tRNA and inhibitory DNA or RNA (RNAi, *e.g.*, small or short hairpin (sh)RNA, microRNA (miRNA), small or short interfering (si)RNA, trans-splicing RNA, or antisense RNA). Polynucleotides include naturally occurring, synthetic, and intentionally modified or altered polynucleotides (*e.g.*, variant nucleic acid). Polynucleotides can be single, double, or triplex, linear or circular, and can be of any length. In discussing polynucleotides, a sequence or structure of a particular

polynucleotide may be described herein according to the convention of providing the sequence in the 5' to 3' direction.

[0156] As used herein, the terms "modify" or "variant" and grammatical variations thereof, mean that a nucleic acid, polypeptide or subsequence thereof deviates from a reference sequence. Modified and variant sequences may therefore have substantially the same, greater or less expression, activity or function than a reference sequence, but at least retain partial activity or function of the reference sequence. Particular examples of a modification or variant are a CpG reduced TTR promoter, ApoE/hAAT promoter, FGG promoter, albumin promoter and SAA1 promoter.

[0157] A "nucleic acid" or "polynucleotide" variant refers to a modified sequence which has been genetically altered compared to wild-type. A nucleic acid or polynucleotide variant can refer to a sequence which has been codon modified but still retains at least partial sequence identity to a reference sequence, such as wild-type sequence. A nucleic acid or polynucleotide that encodes a protein may be genetically modified without altering the encoded protein sequence. Alternatively, the sequence may be genetically modified to encode a variant protein. For example, some codons of such a nucleic acid variant will be changed (*e.g.* CpG reduced) without altering the amino acids of the protein encoded thereby.

[0158] Expression vectors with promoters having reduced CpG content can exhibit improvements compared to promoters in which CpG content has not been reduced. When comparing expression, a CpG reduced promoter is compared to a wild-type or non-CpG reduced promoter.

[0159] The term "variant" or "modified" need not appear in each instance of a reference made to CpG reduced nucleic acid sequence herein. Likewise, the term "CpG reduced nucleic acid" or the like may omit the term "variant" or "modified" but it is intended that reference to "CpG reduced nucleic acid" includes variants at the genetic level.

[0160] A particular example of a variant is a CpG reduced nucleic acid. CpG reduction can be achieved by changing the C or G nucleotide to a different nucleotide, such as changing a C to a T, or changing a G to an A. CpG reduction can also be achieved by deleting a C nucleotide, or deleting a G nucleotide, or deleting both C and G nucleotides.

[0161] A "variant or modified" FVIII refers to a FVIII or FVIII-BDD which has been genetically altered as compared to unmodified wild-type FVIII or FVIII-BDD (SEQ ID NO:68). Such a variant can be referred to as a "nucleic acid variant encoding Factor VIII (FVIII)."

[0162] A "variant Factor VIII (FVIII)" can also mean a modified FVIII protein such that the modified protein has an amino acid alteration compared to wild-type FVIII. When comparing activity and/or stability, if the encoded variant FVIII protein retains the B-domain, it is appropriate to compare it to wild-type FVIII; and if the encoded variant FVIII protein has a B-domain deletion, it is compared to wild-type FVIII that also has a B-domain deletion.

[0163] A variant FVIII can include a portion of the B-domain. Thus, FVIII-BDD includes a portion of the B-domain. Typically, in FVIII-BDD most of the B-domain is deleted.

[0164] A variant FVIII can include an "SQ" sequence set forth as SFSQNPPVLKRHQR (SEQ ID NO:69). Typically, such a variant FVIII with an SQ (FVIII/SQ) has a BDD, *e.g.*, at least all or a part of BD is deleted. Variant FVIII, such as FVIII-BDD can have all or a part of the "SQ" sequence, *i.e.* all or a part of SEQ ID NO:69. Thus, for example, a variant FVIII-BDD with an SQ sequence (SFSQNPPVLKRHQR, SEQ ID NO:69) can have all or just a portion of the amino acid sequence SFSQNPPVLKRHQR. For example, FVIII-BDD can have 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or 13 amino acid residues of SFSQNPPVLKRHQR included. Thus, SFSQNPPVLKRHQR with 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or 13 internal deletions as well as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or 13 amino- or carboxy terminal deletions are included in the variant FVIII proteins set forth herein.

[0165] The "polypeptides," "proteins" and "peptides" encoded by the "nucleic acid" or "polynucleotide" sequences," include full-length native sequences, as with naturally occurring wild-type proteins, as well as functional subsequences, modified forms or sequence variants so long as the subsequence, modified form or variant retain some degree of functionality of the native full-length protein. For example, a nucleic acid (*e.g.*, CpG reduced nucleic acid) encoding FVIII protein can have a B-domain deletion as set forth herein and retain clotting function. In methods and uses of the instant invention, such polypeptides, proteins and peptides encoded by the nucleic acid sequences can be but are not required to be identical to the endogenous protein that is defective, or whose expression is insufficient, or deficient in the treated mammal.

[0166] For example, and without limitation, modifications include one or more nucleotide or amino acid substitutions (*e.g.*, 1-3, 3-5, 5-10, 10-15, 15-20, 20-25, 25-30, 30-40, 40-50, 50-100, 100-150, 150-200, 200-250, 250-500, 500-750, 750-850 or more nucleotides or residues). As set forth herein, an example of a nucleic acid modification is CpG reduction.

[0167] An example of an amino acid modification is a conservative amino acid substitution or a deletion (*e.g.*, subsequences or fragments) of a reference sequence, *e.g.* FVIII, such as FVIII with a B-domain deletion. In certain embodiments, a modified or variant sequence retains at least part of a function or activity of unmodified sequence.

[0168] All mammalian and non-mammalian forms of nucleic acids, including other mammalian forms of the CpG reduced promoters herein are expressly included, either known or unknown.

[0169] The term "vector" refers to small carrier nucleic acid molecule, a plasmid, virus (*e.g.*, AAV), or other vehicle that can be manipulated by insertion or incorporation of a nucleic acid. Vectors can be used for genetic manipulation (*i.e.*, "cloning vectors"), to introduce/transfer polynucleotides into cells and/or organs, and to transcribe or translate the inserted polynucleotide in cells. An "expression vector" is a vector that contains a gene or nucleic acid sequence with the necessary regulatory regions needed for expression in a host cell. A vector nucleic acid sequence generally contains at least an origin of replication for propagation in a cell and optionally additional elements, such as a heterologous nucleic acid sequence, expression control element (*e.g.*, a promoter, enhancer), intron, inverted terminal repeat(s) (ITRs), optional selectable marker, polyadenylation signal.

[0170] As disclosed herein, a vector lacking an intron exhibited superior characteristics compared to the same vector with a synthetic intron. Accordingly, the instant invention provides expression cassettes comprising a transgene operably linked to a regulatory element, wherein the regulatory element (*e.g.,* a CpG reduced promoter as set forth in herein) is positioned upstream of the 5' end of the transgene and in which there are no more than 0 – 5, 5 – 10, 10 – 15, 15 – 20, 20 – 25, 25 – 30, 30 – 35, 35 – 40, 40 – 45, 45 – 50, 50 – 55, 55 – 60, 60 – 65, 65 – 70, 70 – 75, 75 – 80, 80 – 85, 85 – 90, 90 – 95, 95 – 100, 100 – 105, 106 or 107 nucleotides of untranslated nucleic acid sequence between the regulatory element and the 5' end of the transgene.

[0171] The instant invention also provides expression cassettes comprising a first nucleotide sequence having 95% or greater sequence identity to the sequence of any of SEQ ID NOs:2-67, in which the first nucleotide sequence is positioned upstream of the 5' end of a second nucleotide sequence having 95% or greater sequence identity to the sequence of SEQ ID NO:77, and in which no more than 0 – 5, 5 – 10, 10 – 15, 15 – 20, 20 – 25, 25 – 30, 30 – 35, 35 – 40, 40 – 45, 45 – 50, 50 – 55, 55 – 60, 60 – 65, 65 – 70, 70 – 75, 75 – 80, 80 – 85, 85 – 90, 90 – 95, 95 – 100, 100 – 105, 106 or 107 nucleotides of untranslated nucleic acid sequence is between the first nucleotide sequence and the 5' end of the second nucleotide sequence.

[0172] The instant invention additionally provides expression cassettes comprising a first nucleotide sequence having 95% or greater sequence identity to the sequence of any of SEQ ID NOs:2-67, in which the first nucleotide sequence is positioned upstream of the 5' end of a second nucleotide sequence having 95% or greater sequence identity (*e.g.*, 95%, 96%, 97%, 98%, 99% or greater sequence identity) to the sequence of any of SEQ ID NOs:71 – 88, and in which no more than 0 – 5, 5 – 10, 10 – 15, 15 – 20, 20 – 25, 25 – 30, 30 – 35, 35 – 40, 40 – 45, 45 – 50, 50 – 55, 55 – 60, 60 – 65, 65 – 70, 70 – 75, 75 – 80, 80 – 85, 85 – 90, 90 – 95, 95 – 100, 100 – 105, 106 or 107 nucleotides of untranslated nucleic acid sequence is between the first nucleotide sequence and the 5' end of the second nucleotide sequence.

[0173] The instant invention further provides expression cassettes with an untranslated (non-coding) nucleic acid positioned between a regulatory element and a transgene, wherein the untranslated nucleic acid is not an intron. Such an expression cassette can be referred to as an intron-free cassette.

[0174] An intron is a sequence with a donor site and splice acceptor site that allows cellular machinery to splice out untranslated nucleotide sequence during the process of RNA maturation to mRNA. As used herein, "intron-free" refers to an untranslated nucleic acid sequence that lacks donor and splice acceptor sites, but does not mean that the untranslated nucleic acid sequence is devoid of other sites such as restriction enzyme recognition/cleavage sites, Kozak sequences, transcription factor recognition/binding sites. In other words, intron-free does not mean that the nucleic acid sequence is completely devoid of any untranslated nucleic acid sequence(s).

[0175] An AAV vector is derived from adeno-associated virus. AAV vectors are useful as gene therapy vectors as they can penetrate cells and introduce nucleic acid/genetic material so that the nucleic acid/genetic material may be stably maintained in cells. Because AAV is not associated with pathogenic disease in humans, AAV vectors are able to deliver heterologous nucleic acid sequences (*e.g.*, that encode therapeutic proteins and inhibitory RNA) to human patients without causing substantial AAV pathogenesis or disease.

[0176] The term "recombinant," as a modifier of a vector, such as a recombinant AAV (rAAV) vector, as well as a modifier of sequences such as recombinant polynucleotides and polypeptides, means that the compositions have been manipulated (*i.e.*, engineered) in a fashion that generally does not occur in nature. A particular example of a recombinant AAV vector would be where a nucleic acid that is not normally present in the wild-type AAV genome (heterologous sequence) is inserted within the viral genome. An example of would be where a nucleic acid (*e.g.*, gene) encoding a therapeutic protein or polynucleotide sequence is cloned into a vector, with or without 5', 3' and/or intron regions that the gene is normally associated within the AAV genome. Although the term "recombinant" is not always used herein in reference to an AAV vector, as well as sequences such as polynucleotides, recombinant forms including AAV vectors, polynucleotides, etc., are expressly included in spite of any such omission.

[0177] A "rAAV vector" is derived from the wild type genome of AAV by using molecular methods to remove all or a part of the wild type AAV genome, and replacing with a non-native (heterologous) nucleic acid, such as a nucleic acid encoding a therapeutic protein or polynucleotide sequence. Typically, for a rAAV vector one or both inverted terminal repeat (ITR) sequences of AAV genome are retained. A rAAV is distinguished from an AAV genome since all or a part of the AAV genome has been replaced with a non-native sequence with respect to the AAV genomic nucleic acid, such as with a heterologous nucleic acid encoding a therapeutic protein or polynucleotide sequence. Incorporation of a non-native (heterologous) sequence therefore defines the AAV as a "recombinant" AAV vector, which can be referred to as a "rAAV vector."

[0178] A recombinant AAV vector sequence can be packaged- referred to herein as a "particle" for subsequent infection (transduction) of a cell, *ex vivo*, *in vitro* or *in vivo*. Where a recombinant vector sequence is encapsidated or packaged into an AAV particle, the particle

can also be referred to as a "rAAV" or "rAAV particle" or "rAAV virion." Such rAAV, rAAV particles and rAAV virions include proteins that encapsidate or package the vector genome. Particular examples include in the case of AAV, capsid proteins.

[0179] A "vector genome" or conveniently abbreviated as "vg" refers to the portion of the recombinant plasmid sequence that is ultimately packaged or encapsidated to form a rAAV particle. In cases where recombinant plasmids are used to construct or manufacture recombinant AAV vectors, the AAV vector genome does not include the portion of the "plasmid" that does not correspond to the vector genome sequence of the recombinant plasmid. This non vector genome portion of the recombinant plasmid is referred to as the "plasmid backbone," which is important for cloning and amplification of the plasmid, a process that is needed for propagation and recombinant AAV vector production, but is not itself packaged or encapsidated into rAAV particles. Thus, a "vector genome" refers to the nucleic acid that is packaged or encapsidated by rAAV.

[0180] "AAV helper functions" refer to AAV-derived coding sequences (proteins) which can be expressed to provide AAV gene products and AAV vectors that, in turn, function in trans for productive AAV replication and packaging. Thus, AAV helper functions include both of the major AAV open reading frames (ORFs), rep and cap. The Rep expression products have been shown to possess many functions, including, among others: recognition, binding and nicking of the AAV origin of DNA replication; DNA helicase activity; and modulation of transcription from AAV (or other heterologous) promoters. The Cap expression products (capsids) supply necessary packaging functions. AAV helper functions are used to complement AAV functions in trans that are missing from AAV vector genomes.

[0181] An "AAV helper construct" refers generally to a nucleic acid sequence that includes nucleotide sequences providing AAV functions deleted from an AAV vector which is to be used to produce a transducing AVV vector for delivery of a nucleic acid sequence of interest, by way of gene therapy to a subject, for example. AAV helper constructs are commonly used to provide transient expression of AAV rep and/or cap genes to complement missing AAV functions that are necessary for AAV vector replication and encapsidation. Helper constructs generally lack AAV ITRs and can neither replicate nor package themselves. AAV helper constructs can be in the form of a plasmid, phage, transposon, cosmid, virus, or virion. A number of AAV helper constructs have been described, such as plasmids pAAV/Ad and

pIM29+45 which encode both Rep and Cap expression products (See, *e.g.*, Samulski *et al.* (1989) J. Virol. 63:3822-3828; and McCarty *et al.* (1991) J. Virol. 65:2936-2945). A number of other vectors have been described which encode Rep and/or Cap expression products (See, *e.g.*, U.S. Pat. Nos. 5,139,941 and 6,376,237).

[0182] The term "accessory functions" refers to non-AAV derived viral and/or cellular functions upon which AAV is dependent for replication. The term includes proteins and RNAs that are required in AAV replication, including moieties involved in activation of AAV gene transcription, stage specific AAV mRNA splicing, AAV DNA replication, synthesis of Cap expression products and AAV capsid packaging. Viral-based accessory functions can be derived from any of the known helper viruses such as adenovirus, herpesvirus (other than herpes simplex virus type-1) and vaccinia virus.

[0183] An "accessory function vector" refers generally to a nucleic acid molecule that includes polynucleotide sequences providing accessory functions. Such sequences can be on an accessory function vector, and transfected into a suitable host cell. The accessory function vector is capable of supporting rAAV virion production in the host cell. Accessory function vectors can be in the form of a plasmid, phage, transposon or cosmid. In addition, the full-complement of adenovirus genes are not required for accessory functions. For example, adenovirus mutants incapable of DNA replication and late gene synthesis have been reported to be permissive for AAV replication (Ito *et al.*, (1970) J. Gen. Virol. 9:243; Ishibashi *et al.*, (1971) Virology 45:317). Similarly, mutants within E2B and E3 regions have been shown to support AAV replication, indicating that the E2B and E3 regions are probably not involved in providing accessory functions (Carter *et al.*, (1983) Virology 126:505). Adenoviruses defective in the E1 region, or having a deleted E4 region, are unable to support AAV replication. Thus, E1A and E4 regions appear necessary for AAV replication, either directly or indirectly (Laughlin *et al.*, (1982) J. Virol. 41:868; Janik *et al.*, (1981) Proc. Natl. Acad. Sci. USA 78:1925; Carter *et al.*, (1983) Virology 126:505). Other characterized adenovirus mutants include: E1B (Laughlin *et al.* (1982), supra; Janik *et al.*, (1981), supra; Ostrove *et al.*, (1980) Virology 104:502); E2A (Handa *et al.*, (1975) J. Gen. Virol. 29:239; Strauss *et al.*, (1976) J. Virol. 17:140; Myers *et al.*, (1980) J. Virol. 35:665; Jay *et al.*, (1981) Proc. Natl. Acad. Sci. USA 78:2927; Myers *et al.*, (1981) J. Biol. Chem. 256:567); E2B (Carter, Adeno-Associated Virus Helper Functions, in I CRC Handbook of Parvoviruses (P. Tijssen ed., 1990)); E3 (Carter *et al.*, (1983), supra); and E4 (Carter *et al.*, (1983), supra; Carter (1995)).

Studies of the accessory functions provided by adenoviruses having mutations in the E1B coding region have produced conflicting results, but E1B55k may be required for AAV virion production, while E1B19k is not (Samulski *et al.*, (1988) J. Virol. 62:206-210). In addition, International Publication WO 97/17458 and Matshushita *et al.*, (1998) Gene Therapy 5:938-945, describe accessory function vectors encoding various adenovirus genes. Exemplary accessory function vectors comprise an adenovirus VA RNA coding region, an adenovirus E4 ORF6 coding region, an adenovirus E2A 72 kD coding region, an adenovirus E1A coding region, and an adenovirus E1B region lacking an intact E1B55k coding region. Such accessory function vectors are described, for example, in International Publication No. WO 01/83797.

[0184] As used herein, the term "serotype" is a distinction used to refer to an AAV having a capsid that is serologically distinct from other AAV serotypes. Serologic distinctiveness is determined on the basis of the lack of cross-reactivity between antibodies to one AAV as compared to another AAV. Cross-reactivity differences are usually due to differences in capsid protein sequences/antigenic determinants (*e.g.*, due to VP1, VP2, and/or VP3 sequence differences of AAV serotypes).

[0185] Under the traditional definition, a serotype means that the virus of interest has been tested against serum specific for all existing and characterized serotypes for neutralizing activity and no antibodies have been found that neutralize the virus of interest. As more naturally occurring virus isolates of are discovered and/or capsid mutants generated, there may or may not be serological differences with any of the currently existing serotypes. Thus, in cases where the new virus (*e.g.*, AAV) has no serological difference, this new virus (*e.g.*, AAV) would be a subgroup or variant of the corresponding serotype. In many cases, serology testing for neutralizing activity has yet to be performed on mutant viruses with capsid sequence modifications to determine if they are of another serotype according to the traditional definition of serotype. Accordingly, for the sake of convenience and to avoid repetition, the term "serotype" broadly refers to both serologically distinct viruses (*e.g.*, AAV) as well as viruses (*e.g.*, AAV) that are not serologically distinct that may be within a subgroup or a variant of a given serotype.

[0186] rAAV vectors include any viral strain or serotype. For example, and without limitation, a rAAV vector genome or particle (capsid, such as VP1, VP2 and/or VP3) can be

based upon any AAV serotype, such as AAV-1, -2, -3, -4, -5, -6, -7, -8, -9, -10, -11, -12, -rh74, -rh10 or AAV-2i8, for example. Such vectors can be based on the same of strain or serotype (or subgroup or variant), or be different from each other. For example, and without limitation, a rAAV plasmid or vector genome or particle (capsid) based upon one serotype genome can be identical to one or more of the capsid proteins that package the vector. In addition, a rAAV plasmid or vector genome can be based upon an AAV serotype genome distinct from one or more of the capsid proteins that package the vector genome, in which case at least one of the three capsid proteins could be a different AAV serotype, *e.g.*, AAV1, AAV2, AAV3, AAV3B, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, -rh74, -rh10, AAV-2i8, LK03 (SEQ ID NO:91), SPK (SEQ ID NO:92), or variant thereof, for example. More specifically, a rAAV2 vector genome can comprise AAV2 ITRs but capsids from a different serotype, such as AAV1, AAV3, AAV3B, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, -rh74, -rh10, AAV-2i8, LK03 (SEQ ID NO:91), SPK (SEQ ID NO:92) or variant thereof, for example. Accordingly, rAAV vectors include gene/protein sequences identical to gene/protein sequences characteristic for a particular serotype, as well as mixed serotypes also referred to as pseudotypes.

[0187] In certain embodiments, a rAAV vector includes or consists of a capsid sequence at least 70% or more (*e.g.*, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, etc.) identical to one or more AAV1, AAV2, AAV3, AAV3B, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, -rh74, -rh10, AAV-2i8, LK03 (SEQ ID NO:91), SPK (SEQ ID NO:92) capsid proteins (VP1, VP2, and/or VP3 sequences). In certain embodiments, a rAAV vector includes or consists of a sequence at least 70% or more (*e.g.*, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, etc.) identical to one or more AAV1, AAV2, AAV3, AAV3B, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, -rh74, or -rh10 ITR(s).

[0188] In certain embodiments, rAAV vectors include AAV1, AAV2, AAV3, AAV3B, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, Rh10, Rh74 and AAV-2i8 variants (*e.g.*, ITR and capsid variants, such as amino acid insertions, additions, substitutions and deletions) thereof, for example, as set forth in WO 2013/158879 (International Application PCT/US2013/037170), WO 2015/013313 (International Application PCT/US2014/047670) and US 2013/0059732 (US Application No. 13/594,773, discloses LK01, LK02, LK03 (SEQ ID NO:91), etc.).

[0189] rAAV, such as AAV1, AAV2, AAV3, AAV3B, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, -rh74, -rh10, AAV-2i8, LK03 (SEQ ID NO:91), SPK (SEQ ID NO:92) and variants, hybrids and chimeric sequences, can be constructed using recombinant techniques that are known to the skilled artisan, to include one or more heterologous polynucleotide sequences (transgenes) flanked with one or more functional AAV ITR sequences. Such AAV vectors typically retain at least one functional flanking ITR sequence(s), as necessary for the rescue, replication, and packaging of the recombinant vector into a rAAV vector particle. A rAAV vector genome would therefore include sequences required in cis for replication and packaging (*e.g.*, functional ITR sequences).

[0190] As used herein the phrase "bona fide AAV vector" or "bona fide rAAV vector" refers to AAV vectors comprising a heterologous nucleic acid which are capable of infecting target cells. The phrase excludes empty AAV vectors (no heterologous nucleic acid), and AAV vectors lacking full inserts (*e.g.*, heterologous nucleic acid fragments) or those AAV vectors containing host cell nucleic acids.

[0191] The terms "nucleic acid" and "polynucleotide" are used interchangeably herein to refer to all forms of nucleic acid, oligonucleotides, including deoxyribonucleic acid (DNA) and ribonucleic acid (RNA).

[0192] Nucleic acids include genomic DNA, cDNA and antisense DNA, and spliced or unspliced mRNA, rRNA tRNA and inhibitory DNA or RNA (RNAi, *e.g.*, small or short hairpin (sh)RNA, microRNA (miRNA), small or short interfering (si)RNA, trans-splicing RNA, or antisense RNA).

[0193] Nucleic acids include naturally occurring, synthetic, and intentionally modified or altered polynucleotides. Nucleic acids can be single, double, or triplex, linear or circular, and can be of any length. In discussing nucleic acids, a sequence or structure of a particular polynucleotide may be described herein according to the convention of providing the sequence in the 5' to 3' direction.

[0194] A "heterologous" nucleic acid sequence refers to a polynucleotide inserted into an AAV plasmid or vector for purposes of vector mediated transfer/delivery of the polynucleotide into a cell. Heterologous nucleic acid sequences are distinct from AAV nucleic acid, *i.e.*, are non-native with respect to AAV nucleic acid. Once

transferred/delivered into the cell, a heterologous nucleic acid sequence, contained within the vector, can be expressed (*e.g.*, transcribed, and translated if appropriate). Alternatively, a transferred/delivered heterologous polynucleotide in a cell, contained within the vector, need not be expressed. Although the term "heterologous" is not always used herein in reference to nucleic acid sequences and polynucleotides, reference to a nucleic acid sequence or polynucleotide even in the absence of the modifier "heterologous" is intended to include heterologous nucleic acid sequences and polynucleotides in spite of the omission.

[0195] A "transgene" is used herein to conveniently refer to a nucleic acid that is intended or has been introduced into a cell or organism. Transgenes include any nucleic acid, such as a heterologous nucleic acid encoding a therapeutic protein or polynucleotide sequence. The term transgene and heterologous nucleic acid/polynucleotide sequences are used interchangeably herein.

[0196] In a cell having a transgene, the transgene has been introduced/transferred by way of a plasmid or a AAV vector, "transduction" or "transfection" of the cell. The terms "transduce" and "transfect" refer to introduction of a molecule such as a nucleic acid into a host cell (*e.g.*, HEK293) or cells or organ of an organism. The transgene may or may not be integrated into genomic nucleic acid of the recipient cell.

[0197] The "nucleic acids," "polynucleotides," "heterologous nucleic acids," "transgenes" and "CpG reduced nucleic acid sequences" include full-length sequences, as well as functional subsequences, so long as the subsequence, retains some degree of functionality of the full-length sequence. Nucleic acids, polynucleotides, heterologous nucleic acids, transgenes and CpG reduced nucleic acid sequences.

[0198] The "polypeptides," "proteins" and "peptides" encoded by the "nucleic acid sequence" such as a heterologous nucleic acid sequence include full-length sequences, as with naturally occurring proteins, as well as functional subsequences, modified forms or sequence variants so long as the subsequence, modified form or variant retains some degree of functionality of the full-length protein. Such polypeptides, proteins and peptides encoded by the nucleic acid sequences can be but are not required to be identical to the endogenous protein that is defective, or whose expression is insufficient, or deficient in the treated mammal.

[0199] A “host cell" denotes, for example, microorganisms, yeast cells, insect cells, and mammalian cells, that can be, or have been, used as recipients of an AAV vector plasmid, AAV helper construct, an accessory function vector, or other transfer DNA. The term includes the progeny of the original cell which has been transfected. Thus, a "host cell" generally refers to a cell which has been transfected with an exogenous DNA sequence. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation. Exemplary host cells include human embryonic kidney (HEK) cells such as HEK293.

[0200] A “transduced cell” is a cell into which a transgene has been introduced. Accordingly, a “transduced” cell means a genetic change in a cell following incorporation of an exogenous molecule, for example, a nucleic acid (*e.g.*, a transgene) into the cell. Thus, a “transduced” cell is a cell into which, or a progeny thereof in which an exogenous nucleic acid has been introduced. The cell(s) can be propagated (cultured) and the introduced protein expressed or nucleic acid transcribed, or vector, such as rAAV, produced by the cell. For gene therapy uses and methods, a transduced cell can comprise an organ or tissue and in turn can be in a subject.

[0201] As used herein, the term “stable” in reference to a cell, or "stably integrated" means that nucleic acid sequences, such as a selectable marker or heterologous nucleic acid sequence, or plasmid or vector has been inserted into a chromosome (*e.g.*, by homologous recombination, non-homologous end joining, transfection, etc.) or is maintained in the recipient cell or host organism extrachromosomally, and has remained in the chromosome or is maintained extrachromosomally for a period of time.

[0202] A “cell line” refers to a population of cells capable of continuous or prolonged growth and division *in vitro* under appropriate culture conditions. Cell lines can, but need not be, clonal populations derived from a single progenitor cell. In cell lines, spontaneous or induced changes can occur in karyotype during storage or transfer of such clonal populations, as well as during prolonged passaging in tissue culture. Thus, progeny cells derived from the cell line may not be precisely identical to the ancestral cells or cultures. An exemplary cell line applicable to the instant invention purification methods is HEK293.

[0203] An "expression control element" refers to nucleic acid sequence(s) that influence expression of an operably linked nucleic acid. Control elements, including expression control elements as set forth herein such as promoters and enhancers. rAAV vectors can include one or more "expression control elements." Typically, such elements are included to facilitate proper heterologous polynucleotide transcription and if appropriate translation (*e.g.*, one or more of a promoter, enhancer, splicing signal for introns, maintenance of the correct reading frame of the gene to permit in-frame translation of mRNA and, stop codons, etc.). Such elements typically act in cis, referred to as a "cis acting" element, but may also act in trans.

[0204] Expression control can be effected at the level of transcription, translation, splicing, message stability, etc. Typically, an expression control element that modulates transcription is juxtaposed near the 5' end (*i.e.*, "upstream") of a transcribed nucleic acid. Expression control elements can also be located at the 3' end (*i.e.*, "downstream") of the transcribed sequence or within the transcript (*e.g.*, in an intron). Expression control elements (*e.g.*, CpG reduced TTR, ApoE/hAAT, FGG, albumin, and SAA1 promoters as well as fusions/hybrids thereof) can be located adjacent to or at a distance away from the transcribed sequence (*e.g.*, 1-10, 10-25, 25-50, 50-100, 100 to 500, or more nucleotides from the polynucleotide), even at considerable distances from the 5' or 3' end. Nevertheless, owing to the length limitations of rAAV vectors, expression control elements will typically be within 1 to 1000 nucleotides from the transcribed nucleic acid.

[0205] Functionally, expression of operably linked nucleic acid is at least in part controllable by the element (*e.g.*, promoter, enhancer, etc.) such that the element modulates transcription of the nucleic acid and, as appropriate, translation of the transcript. A specific example of an expression control element is a promoter, which is usually located 5' of the transcribed sequence. A promoter typically increases expression from operably linked nucleic acid as compared to an amount (if any) expressed when no promoter exists.

[0206] Examples of promoters include TTR and ApoE/hAAT promoters, including CpG reduced versions and hybrid forms of the TTR promoter disclosed herein. Further examples of promoters include ApoE/hAAT, FGG, albumin, and SAA1 promoters including CpG reduced versions and hybrid forms thereof.

[0207] An "enhancer" as used herein can refer to a sequence that is located adjacent to the nucleic acid sequence, such as a heterologous nucleic acid sequence. Enhancer elements are

typically located upstream (5') of a promoter element but also function and can be located downstream (3') of or within a sequence. Hence, an enhancer element can be located upstream or downstream, *e.g.*, within 100 base pairs, 200 base pairs, or 300 or more base pairs of the as selectable marker, and/or a heterologous nucleic acid encoding a therapeutic protein or polynucleotide sequence. Enhancer elements typically increase expression of an operably linked nucleic acid above expression afforded by a promoter element.

[0208] The term "operably linked" means that the regulatory sequences necessary for expression of a nucleic acid sequence are placed in the appropriate positions relative to the sequence so as to effect expression of the nucleic acid sequence. This same definition is sometimes applied to the arrangement of nucleic acid sequences and transcription control elements (*e.g.* promoters, enhancers, and termination elements) in an expression vector, *e.g.*, rAAV vector.

[0209] In the example of an expression control element in operable linkage with a nucleic acid, the relationship is such that the control element modulates expression of the nucleic acid. More specifically, for example, two DNA sequences operably linked means that the two DNAs are arranged (cis or trans) in such a relationship that at least one of the DNA sequences is able to exert a modulatory effect upon the other sequence.

[0210] Accordingly, additional elements for vectors include, without limitation, an expression control (*e.g.*, promoter/enhancer) element, a transcription termination signal or stop codon, 5' or 3' untranslated regions (*e.g.*, polyadenylation (polyA) sequences) which flank a sequence (*e.g.*, heterologous sequence), such as one or more copies of an AAV ITR sequence, or an intron.

[0211] Further elements include, for example, filler or stuffer polynucleotide sequences, for example to improve packaging and reduce the presence of contaminating nucleic acid. AAV vectors typically accept inserts of DNA having a size range which is generally about 4 kb to about 5.2 kb, or slightly more. Thus, for shorter sequences, inclusion of a stuffer or filler in order to adjust the length to near or at the normal size of the virus genomic sequence acceptable for vector packaging into a rAAV particle. In certain embodiments, a filler/stuffer nucleic acid sequence is an untranslated (non-protein encoding) segment of nucleic acid. For a nucleic acid sequence less than 4.7 Kb, the filler or stuffer polynucleotide sequence has a

length that when combined (*e.g.*, inserted into a vector) with the sequence has a total length between about 3.0-5.5Kb, or between about 4.0-5.0Kb, or between about 4.3-4.8Kb.

[0212] Where a wild type heterologous nucleic acid or transgene is too large to be packaged within an AAV vector particle, the heterologous nucleic acid may be provided in modified, fragmented or truncated form for packaging in and delivery by an AAV vector, such that a functional protein or nucleic acid product, such as a therapeutic protein or nucleic acid product, is ultimately provided.

[0213] In certain embodiments, the heterologous nucleic acid that encodes a protein (*e.g.*, therapeutic protein) is provided in modified or truncated forms or the heterologous nucleic acid is provided in multiple constructs, delivered by separate and multiple AAV vectors.

[0214] In certain embodiments, the heterologous nucleic acid is provided as a truncated variant that maintains functionality of the encoded protein (*e.g.*, therapeutic protein), including removal of portions unnecessary for function, such that the encoding heterologous polynucleotide is reduced in size for packaging in an AAV vector.

[0215] In certain embodiments the heterologous nucleic acid is provided in split AAV vectors, each providing nucleic acid encoding different portions of a protein (*e.g.*, therapeutic protein), thus delivering multiple portions of a protein (*e.g.*, therapeutic protein) which assemble and function in the cell.

[0216] In certain embodiments, the heterologous nucleic acid is provided by dual AAV vectors using overlapping, trans-splicing or hybrid trans-splicing dual vector technology. In certain embodiments, two overlapping AAV vectors are used which combine in the cell to generate a full expression cassette, from which a full-length protein (*e.g.*, therapeutic protein) is expressed.

[0217] The term "identity," "homology" and grammatical variations thereof, mean that two or more referenced entities are the same, when they are "aligned" sequences. Thus, by way of example, when two polypeptide sequences are identical, they have the same amino acid sequence, at least within the referenced region or portion. Where two polynucleotide sequences are identical, they have the same polynucleotide sequence, at least within the referenced region or portion. The identity can be over a defined area (region or domain) of

the sequence. An "area" or "region" of identity refers to a portion of two or more referenced entities that are the same. Thus, where two protein or nucleic acid sequences are identical over one or more sequence areas or regions they share identity within that region. An "aligned" sequence refers to multiple polynucleotide or protein (amino acid) sequences, often containing corrections for missing or additional bases or amino acids (gaps) as compared to a reference sequence.

[0218] The identity can extend over the entire length or a portion of the sequence. In certain embodiments, the length of the sequence sharing the percent identity is 2, 3, 4, 5 or more contiguous nucleic acids or amino acids, *e.g.*, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, etc. contiguous nucleic acids or amino acids. In certain embodiments, the length of the sequence sharing identity is 21 or more contiguous nucleic acids or amino acids, *e.g.*, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, etc. contiguous nucleic acids or amino acids. In certain embodiments, the length of the sequence sharing identity is 41 or more contiguous nucleic acids or amino acids, *e.g.*, 42, 43, 44, 45, 45, 47, 48, 49, 50, etc., contiguous nucleic acids or amino acids. In certain embodiments, the length of the sequence sharing identity is 50 or more contiguous nucleic acids or amino acids, *e.g.*, 50-55, 55-60, 60-65, 65-70, 70-75, 75-80, 80-85, 85-90, 90-95, 95-100, 100-150, 150-200, 200-250, 250-300, 300-500, 500-1,000, etc. contiguous nucleic acids or amino acids.

[0219] As set forth herein, nucleic acid variants such as CpG reduced promoters including hybrid forms thereof will be distinct from wild-type but may exhibit sequence identity with wild-type promoters. In CpG reduced promoters including hybrid forms thereof, at the nucleotide sequence level, a CpG reduced promoter will typically be at least about 70% identical, more typically at least about 75% identical, even more typically about 80%-90% identical to wild-type promoter. For example, a CpG reduced promoter may have 70%-99% identity to wild-type promoter. Accordingly, a CpG reduced promoter may have 70 – 75%, 75 – 80%, 80 – 85%, 85 – 90%, 90 – 95%, 95 – 99%, 75%-99% identity to wild-type promoter.

[0220] At the amino acid sequence level, a variant such as a variant FVIII or hFVIII-BDD protein will be at least about 70% identical, more typically about 75% identical, or about 80% identical, even more typically about 85 identical, or about 90% or more identical to a reference sequence. In certain embodiments, a variant such as a variant FVIII or hFVIII-

BDD protein has at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identity to a reference sequence, *e.g.,* wild-type FVIII protein with or without B-domain.

[0221] The terms "homologous" or "homology" mean that two or more referenced entities share at least partial identity over a given region or portion. "Areas, regions or domains" of homology or identity mean that a portion of two or more referenced entities share homology or are the same. Thus, where two sequences are identical over one or more sequence regions they share identity in these regions. "Substantial homology" means that a molecule is structurally or functionally conserved such that it has or is predicted to have at least partial structure or function of one or more of the structures or functions (*e.g.,* a biological function or activity) of the reference molecule, or relevant/corresponding region or portion of the reference molecule to which it shares homology.

[0222] The extent of identity (homology) or "percent identity" between two sequences can be ascertained using a computer program and/or mathematical algorithm. For purposes of this invention comparisons of nucleic acid sequences are performed using the GCG Wisconsin Package version 9.1, available from the Genetics Computer Group in Madison, Wisconsin. 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. For polypeptide sequence comparisons, a BLASTP algorithm is typically used in combination with a scoring matrix, such as PAM100, PAM 250, BLOSUM 62 or BLOSUM 50. FASTA (*e.g.*, FASTA2 and FASTA3) and SSEARCH sequence comparison programs are also used to quantitate extent of identity (Pearson *et al.*, *Proc. Natl. Acad. Sci.* USA 85:2444 (1988); Pearson, *Methods Mol Biol.* 132:185 (2000); and Smith *et al.*, *J. Mol. Biol.* 147:195 (1981)). Programs for quantitating protein structural similarity using Delaunay-based topological mapping have also been developed (Bostick *et al.*, *Biochem Biophys Res Commun.* 304:320 (2003)).

[0223] A "therapeutic protein," in certain embodiments, is a peptide or protein that may alleviate or reduce symptoms that result from an insufficient amount, absence or defect in a protein in a cell or subject. A "therapeutic" protein encoded by a transgene can confer a benefit to a subject, *e.g.*, to correct a genetic defect, to correct a gene (loss of expression or function) deficiency, etc.

[0224] For example, and without limitation, heterologous nucleic acids encoding gene products (*e.g.*, therapeutic proteins) useful in accordance with the instant invention include those that may be used in the treatment of a disease or disorder including, but not limited to, "hemostasis" or blood clotting (bleeding) disorders such as hemophilia A, hemophilia A patients with inhibitory antibodies, hemophilia B, hemophilia B with inhibitory antibodies, a deficiency in any blood coagulation Factor: VII, VIII, IX, X, XI, V, XII, II, von Willebrand factor, combined FV/FVIII deficiency, thalassemia, vitamin K epoxide reductase C1 deficiency, gamma-carboxylase deficiency; anemia; bleeding associated with trauma, injury, thrombosis, thrombocytopenia, stroke, coagulopathy, disseminated intravascular coagulation (DIC); over-anticoagulation associated with heparin, low molecular weight heparin, pentasaccharide, warfarin, small molecule antithrombotics (*i.e.,* FXa inhibitors); and platelet disorders such as, Bernard Soulier syndrome, Glanzmann thrombasthenia, and storage pool deficiency. In certain embodiments, a subject has a blood clotting disorder. In certain embodiments, a subject has hemophilia A, hemophilia A with inhibitory antibodies, hemophilia B, hemophilia B with inhibitory antibodies, a deficiency in any coagulation Factor: VII, VIII, IX, X, XI, V, XII, II, von Willebrand factor, or a combined FV/FVIII deficiency, thalassemia, vitamin K epoxide reductase C1 deficiency or gamma-carboxylase deficiency.

[0225] In certain embodiments, a subject has a disease or disorder including, for example and without limitation, a lung disease (*e.g.*, cystic fibrosis), a bleeding disorder (*e.g.*, hemophilia A or hemophilia B with or without inhibitors), thalassemia, a blood disorder (*e.g.*, anemia), a neurodegenerative disorder (*e.g.*, Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS)), a neurological disorder (*e.g.*, epilepsy), a lysosomal storage disease)*e.g.*, aspartylglucosaminuria, Batten disease, late infantile neuronal ceroid lipofuscinosis type 2 (CLN2), cystinosis, Fabry disease, Gaucher disease types I, II, and III, glycogen storage disease II (Pompe disease), glycogen storage disease III (GSDIII; Cori disease); ganglioside monosialic 2 (GM2)-gangliosidosis type I

(Tay Sachs disease), GM2-gangliosidosis type II (Sandhoff disease), mucolipidosis types I (sialidosis type I and II), II (I-cell disease), III (pseudo-Hurler disease) and IV, mucopolysaccharide storage diseases (Hurler disease and variants, Hunter, Sanfilippo Types A,B,C,D, Morquio Types A and B, Maroteaux-Lamy and Sly diseases), Niemann-Pick disease types A/B, C1 and C2, and Schindler disease types I and II), an inflammatory disorder (*e.g.*, hereditary angioedema (HAE)), a copper or iron accumulation disorder (*e.g.*, Wilson's or Menkes disease), lysosomal acid lipase deficiency, cancer, type 1 or type 2 diabetes, adenosine deaminase deficiency, a metabolic disease or disorder (*e.g.*, glycogen storage diseases, methylmalonic acidemia, ornithine transcarbamylase deficiency, hypophosphatsia, very-long-chain acyl-CoA dehydrogenase deficiency (VLCAD), galactosemia), an autoimmune disease (*e.g.*, multiple sclerosis, type I diabetes, celiac disease, neuromyelitis optica (NMO), immune thrombocytopenia ((ITP); idiopathic thrombocytopenic purpura), Addison's disease, myasthenia gravis), a disease of solid organs (*e.g.*, brain, liver, kidney, heart), or an infectious viral (*e.g.*, hepatitis B and C, human immunodeficiency virus (HIV), etc.), bacterial or fungal disease.

[0226] In certain embodiments, a subject has a disease that affects or originates in the central nervous system (CNS). In certain embodiments, the disease is a neurodegenerative disease. In certain embodiments, the CNS or neurodegenerative disease is Alzheimer's disease, Huntington's disease, ALS, hereditary spastic hemiplegia, primary lateral sclerosis, spinal muscular atrophy, Kennedy's disease, a polyglutamine repeat disease, or Parkinson's disease. In certain embodiments, the CNS or neurodegenerative disease is a polyglutamine repeat disease. In certain embodiments, the polyglutamine repeat disease is a spinocerebellar ataxia (SCA1, SCA2, SCA3, SCA6, SCA7, or SCA17).

[0227] Apolipoprotein E (ApoE) is a major cholesterol carrier involved in lipid transport and brain injury repair. It is suggested that human ApoE isoforms differentially affect the clearance or synthesis of amyloid-β (Aβ) *in vivo*. The epsilon4 (ε4) allele of ApoE is associated with increased risk of Alzheimer's disease (AD) , and the presence of the ApoE ε2 allele appears to decrease AD risk, and is a protective ApoE isoform. As used herein, the term "protective ApoE isoform" refers to ApoE isoforms that decrease one or more symptoms or indications of Alzheimer's disease (*e.g.*, physical, physiological, biochemical, histological, behavioral). A protective ApoE isoform also refers to ApoE isoforms that can reduce the risk of Alzheimer's

disease by at least 5%, such as 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% or more.

[0228] In certain embodiments, the invention provides a method of delivering a protective ApoE isoform (*e.g.*, ApoE ε2) to the CNS of a subject (*e.g.*, mammal), by way of delivery or administration to a non-CNS cell, organ or tissue (*e.g.*, not to cerebrospinal fluid (CSF) or brain) of the subject.

[0229] In certain embodiments, an rAAV particle comprising an AAV capsid protein and a vector comprising a nucleic acid encoding a protective ApoE isoform (*e.g.*, ApoE ε2) inserted between a pair of AAV inverted terminal repeats (ITRs) in a manner effective to transduce non-CNS cells (*e.g.*, liver cells) in a subject (*e.g.*, mammal) such that the non-CNS cells (*e.g.*, liver cells) secrete the protective ApoE isoform into the systemic circulation (vasculature or blood vessels) of the subject. The protective ApoE isoform in the circulation crosses the blood brain barrier and enters the CNS (*e.g.*, cerebrospinal fluid (CSF) or brain, such as brain parenchyma).

[0230] In certain embodiments, the instant invention provides a vector, expression cassette or nucleic acid that encodes a protective ApoE isoform (*e.g.*, ApoE ε2) that is expressed in the liver or in liver cells.

[0231] In certain embodiments, the heterologous nucleic acid encodes a protein selected from the group consisting of insulin, glucagon, growth hormone (GH), parathyroid hormone (PTH), growth hormone releasing factor (GRF), follicle stimulating hormone (FSH), luteinizing hormone (LH), human chorionic gonadotropin (hCG), vascular endothelial growth factor (VEGF), angiopoietins, angiostatin, granulocyte colony stimulating factor (GCSF), erythropoietin (EPO), connective tissue growth factor (CTGF), basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), epidermal growth factor (EGF), transforming growth factor α (TGFα), platelet-derived growth factor (PDGF), insulin growth factors I and II (IGF-I and IGF-II), TGFβ, activins, inhibins, bone morphogenic protein (BMP), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophins NT-3 and NT4/5, ciliary neurotrophic factor (CNTF), glial cell line derived neurotrophic factor (GDNF), neurturin, agrin, netrin-1 and netrin-2, hepatocyte growth factor (HGF), ephrins, noggin, sonic hedgehog and tyrosine hydroxylase.

[0232] In certain embodiments, the heterologous nucleic acid encodes a protein selected from the group consisting of thrombopoietin (TPO), interleukins (IL1 through IL-36),

monocyte chemoattractant protein, leukemia inhibitory factor, granulocyte-macrophage colony stimulating factor, Fas ligand, tumor necrosis factors α and β, interferons α, β, and γ, stem cell factor, flk-2/flt3 ligand, IgG, IgM, IgA, IgD and IgE, chimeric immunoglobulins, humanized antibodies, single chain antibodies, T cell receptors, chimeric T cell receptors, single chain T cell receptors, class I and class II MHC molecules.

[0233] In certain embodiments, the heterologous nucleic acid encodes CFTR (cystic fibrosis transmembrane regulator protein), a blood coagulation (clotting) factor (Factor XIII, Factor IX, Factor VIII, Factor X, Factor VII, Factor VIIa, protein C, etc.), a gain of function blood coagulation factor, an antibody, retinal pigment epithelium-specific 65 kDa protein (RPE65), erythropoietin, LDL receptor, lipoprotein lipase, ornithine transcarbamylase, β-globin, α-globin, spectrin, α-antitrypsin, adenosine deaminase (ADA), a metal transporter (ATP7A or ATP7), sulfamidase, an enzyme involved in lysosomal storage disease (ARSA), hypoxanthine guanine phosphoribosyl transferase, β-25 glucocerebrosidase, sphingomyelinase, lysosomal hexosaminidase, branched-chain keto acid dehydrogenase, a hormone, a growth factor, insulin-like growth factor 1 or 2, platelet derived growth factor, epidermal growth factor, nerve growth factor, neurotrophic factor -3 and -4, brain-derived neurotrophic factor, glial derived growth factor, transforming growth factor α and β, a cytokine, α-interferon, β-interferon, interferon-γ, interleukin-2, interleukin-4, interleukin 12, granulocyte-macrophage colony stimulating factor, lymphotoxin, a suicide gene product, herpes simplex virus thymidine kinase, cytosine deaminase, diphtheria toxin, cytochrome P450, deoxycytidine kinase, tumor necrosis factor, a drug resistance protein, a tumor suppressor protein (*e.g.*, p53, Rb, Wt-1, NF1, Von Hippel–Lindau (VHL), adenomatous polyposis coli (APC)), a peptide with immunomodulatory properties, a tolerogenic or immunogenic peptide or protein Tregitope or hCDR1, insulin, glucokinase, guanylate cyclase 2D (LCA-GUCY2D), Rab escort protein 1 (choroideremia), LCA 5 (LCA-lebercilin), ornithine ketoacid aminotransferase (gyrate atrophy), retinoschisin 1 (X-linked retinoschisis), USH1C (Usher's Syndrome 1C), X-linked retinitis pigmentosa GTPase (XLRP), MERTK (AR forms of RP: retinitis pigmentosa), DFNB1 (connexin 26 deafness), ACHM 2, 3 and 4 (achromatopsia), PKD-1 or PKD-2 (polycystic kidney disease), TPP1, CLN2, a sulfatase, N-acetylglucosamine-1-phosphate transferase, cathepsin A, GM2-AP, Niemann-Pick C1 (NPC1), VPC2, a sphingolipid activator protein, one or more zinc finger nuclease for genome editing, and one or more donor sequence used as repair templates for genome editing.

[0234] In certain embodiments, the protein encoded by the heterologous nucleic acid comprises a gene editing nuclease. In certain embodiments, the gene editing nuclease comprises a zinc finger nuclease (ZFN) or a transcription activator-like effector nuclease (TALEN). In certain embodiments, the gene editing nuclease comprises a functional Type II CRISPR-Cas9.

[0235] Other heterologous nucleic acids encoding gene products (*e.g.*, therapeutic proteins) that may be used with the instant invention, and which may optionally be expressed in liver or liver cells (*e.g.*, hepatocytes) and provide a benefit, include, for example and without limitation: GAA (acid alpha-glucosidase) for treatment of Pompe disease; ATP7B (copper transporting ATPase2) for treatment of Wilson's disease; alpha galactosidase (GLA) for treatment of Fabry disease; ASS1 (arginosuccinate synthase) for treatment of citrullinemia type 1; beta-glucocerebrosidase for treatment of Gaucher disease Type 1; beta-hexosaminidase A for treatment of Tay Sachs disease; SERPING1 (C1 protease inhibitor; C1 esterase inhibitor (C1EI)) for treatment of hereditary angioedema (HAE); glucose-6-phosphatase for treatment of glycogen storage disease type I (GSDI); glycogen-debranching enzyme (GDE) for treatment of glycogen storage disease type III (GSD III; cori disease); Niemann-Pick C1 protein (NPC intracellular cholesterol transporter 1; NPC1) for treatment of Niemann-Pick disease; erythropoietin (EPO) for treatment of anemia; interferon-alpha, interferon-beta, and interferon-gamma for treatment of various immune disorders, viral infections and cancer; an interleukin (IL), including any one of IL-1 through IL-36, and corresponding receptors, for treatment of various inflammatory diseases or immuno-deficiencies; a chemokine, including chemokine (C-X-C motif) ligand 5 (CXCL5) for treatment of immune disorders; granulocyte-colony stimulating factor (G-CSF) for treatment of immune disorders such as Crohn's disease; granulocyte-macrophage colony stimulating factor (GM-CSF) for treatment of various human inflammatory diseases; macrophage colony stimulating factor (M-CSF) for treatment of various human inflammatory diseases; keratinocyte growth factor (KGF) for treatment of epithelial tissue damage; chemokines such as monocyte chemoattractant protein-1 (MCP-1) for treatment of recurrent miscarriage, HIV-related complications, and insulin resistance; tumor necrosis factor (TNF) and receptors for treatment of various immune disorders; alpha1-antitrypsin for treatment of emphysema or chronic obstructive pulmonary disease (COPD); alpha-L-iduronidase for treatment of mucopolysaccharidosis I (MPS I); ornithine transcarbamoylase (OTC) for treatment of OTC deficiency; phenylalanine hydroxylase (PAH) or phenylalanine ammonia-lyase (PAL) for

treatment of phenylketonuria (PKU); lipoprotein lipase for treatment of lipoprotein lipase deficiency; apolipoproteins for treatment of apolipoprotein (Apo) A-I deficiency; low-density lipoprotein receptor (LDL-R) for treatment of familial hypercholesterolemia (FH); albumin for treatment of hypoalbuminemia; lecithin cholesterol acyltransferase (LCAT); carbamoyl synthetase I; argininosuccinate synthetase; argininosuccinate lyase; arginase; fumarylacetoacetate hydrolase; porphobilinogen deaminase; cystathionine beta-synthase, for treatment of homocystinuria; branched chain ketoacid decarboxylase; isovaleryl-CoA dehydrogenase; propionyl CoA carboxylase; methylmalonyl-CoA mutase; glutaryl CoA dehydrogenase; insulin; pyruvate carboxylase; hepatic phosphorylase; phosphorylase kinase; glycine decarboxylase; H-protein; T-protein; cystic fibrosis transmembrane regulator (CFTR); ATP-binding cassette, sub-family A (ABC1), member 4 (ABCA4) for the treatment of Stargardt disease; and dystrophin.

[0236] In certain embodiments a subject has an autoimmune disease or disorder (*e.g.*, multiple sclerosis, anti-MAG peripheral neuropathy, type 1 diabetes, Graves' disease, rheumatoid arthritis, proteoglycan induced arthritis (PGIA) or myasthenia gravis); an allergy or allergic disease.

[0237] Mature myelin oligodendrocyte glycoprotein (MOG) is associated with the bi-lipid layer. MOG is characterized by an IgV-like extracellular domain, a single-bypass transmembrane protein, a membrane-associated domain, and a cytoplasmic tail. The extracellular IgV-like domain is denoted herein as mini-MOG (mMOG). MOG is predominantly found in membranes of oligodendrocytes and contributes a small amount to the final composition of myelin. Autoimmune responses to MOG are implicated in the development and etiology of multiple sclerosis.

[0238] In certain embodiments, the therapeutic protein is a fusion protein comprising an unwanted antigen and a leader sequence for cell secretion.

[0239] In certain embodiments, the therapeutic protein is a fusion protein comprising the extracellular domain of MOG, or a fragment thereof, and a leader sequence for cell secretion.

[0240] In certain embodiments, an expression cassette comprises an regulatory element operably linked to a nucleic acid encoding a fusion protein comprising an unwanted antigen and a leader sequence for cell secretion.

[0241] In certain embodiments, the unwanted antigen comprises a self-antigen, autoantigen or protein or peptide that has structural similarity or sequence identity to the self-antigen or the autoantigen. In certain embodiments, the protein or peptide that has structural similarity or sequence identity to the self-antigen or the autoantigen is a microbial protein or peptide. In certain embodiments, the unwanted antigen comprises an allergen.

[0242] In certain embodiments, the allergen comprises a plant, insect, or animal allergen. In certain embodiments, the unwanted antigen comprises a myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), proteolipid protein (PLP), or subsequence thereof.

[0243] In certain embodiments, the MOG lacks all or a part of its transmembrane domain. In certain embodiments, the MOG comprises or consists of amino acids 1 – 117 of mature MOG. In certain embodiments, the MOG subsequence is a subsequence of its extracellular domain or a subsequence of its transmembrane domain. In certain embodiments, the MOG comprises or consists of amino acids 35 – 55, 118 – 132, 181 – 195, or 186 – 200 of mature MOG. In certain embodiments, the MOG comprises or consists of amino acids 1 – 20, 11 – 30, 21 – 40, 31 – 50, etc. of mature MOG.

[0244] In certain embodiments, the instant invention provides methods of suppressing, reducing or inhibiting a cell-mediated or antibody mediated immune response to an unwanted antigen in a mammal. In certain embodiment, a method includes providing an expression cassette, particle or pharmaceutical composition or LNP composition as set forth herein; and administering an amount of the expression cassette, particle, pharmaceutical composition or LNP composition to the mammal, wherein the fusion protein is expressed in the mammal sufficient to suppress, reduce or inhibit a cell-mediated or antibody mediated immune response to the unwanted antigen.

[0245] In certain embodiments, the instant invention provides methods of inducing tolerance in a mammal to an unwanted antigen. In certain embodiments, a method includes providing an expression cassette, particle, or pharmaceutical composition or LNP composition as set forth herein; and administering an amount of the expression cassette, particle, pharmaceutical or LNP composition to the mammal, wherein the fusion protein is expressed in the mammal sufficient to induce tolerance to the unwanted antigen.

[0246] In certain embodiments, the instant invention provides methods of treating a subject (*e.g.*, human) in need of a fusion protein. In certain embodiments, a method includes providing an expression cassette, particle, or pharmaceutical composition or LNP composition as set forth herein; and administering an amount of the expression cassette, particle, pharmaceutical or LNP composition to the subject (*e.g.*, human), wherein the fusion protein is expressed in the subject (*e.g.*, human).

[0247] In certain embodiments, the subject (*e.g.*, human) has an autoimmune disease or disorder. In certain embodiments, the subject (*e.g.*, human) has an allergy or allergic disease or disorder.

[0248] In certain embodiments, the subject (*e.g.*, human) has multiple sclerosis, anti-MAG peripheral neuropathy, type 1 diabetes, Graves' disease, rheumatoid arthritis, proteoglycan induced arthritis (PGIA) or myasthenia gravis.

[0249] As used herein, an "unwanted antigen" is a self – antigen or autoantigen that is able to induce, provide, enhance and/or stimulate immune tolerance against the antigen itself or a protein that includes all or a portion of the antigen and/or that suppresses, inhibits, reduces and/or decreases an immune response directed towards the antigen itself or a protein that includes all or a portion of the antigen. An unwanted antigen as used herein also includes allergens or allergenic antigens that can induce, provide, enhance and/or stimulate immune tolerance against the allergen as well as allergens and allergenic antigens that suppress, inhibit, reduce and/or decrease an immune response directed towards the allergen or an entity that includes the allergen.

[0250] Unwanted antigens as set forth herein also include allogenic antigens or transplantation antigens or minor histocompatibility antigens that can lead to rejection of a cell, tissue or organ after their transplantation into a subject. The subject typically recognizes the transplanted cell, tissue or organ as foreign and develops an immune response against the cell, tissue or organ. Accordingly, the invention methods are directed to preventing or reducing rejection of a cell, tissue or organ after transplant into a subject.

[0251] Although not wishing to be bound by any theory or particular mechanism, it is believed that the unwanted antigen functions by binding to or activating T regulatory cells (Tregs) thereby preventing, suppressing, inhibiting, reducing, decreasing or otherwise down

regulating an immune response. This binding to or activation of Tregs in turn can lead to immune tolarization against the self – antigen or autoantigen.

[0252] As used herein, a "leader" sequence is an amino acid sequence that when linked to a protein provides or facilitates secretion of the linked protein from the cell in which it is expressed. A leader sequence as used herein can also be referred to as a secretion sequence. Such leader and secretion sequences are intended to provide or facilitate cell secretion but may not always facilitate secretion if they are linked to a protein that has a signal sequence that may prevent secretion of the protein.

[0253] In certain embodiments, an unwanted antigen comprises an autoimmune disease protein or a subsequence thereof. An autoimmune disease protein includes any antigen (such as a protein, subsequence thereof, or a peptide) that contributes to initiation and/or progression of an autoimmune disease. Such autoimmune disease proteins can be derived from other organisms, such as microorganisms because the sequence or structure of the proteins from the other organisms mimic the self – antigen or autoantigen.

[0254] In certain embodiments, an autoimmune disease protein is myelin oligodendrocyte glycoprotein (MOG, *e.g.*, for multiple sclerosis), myelin basic protein (MBP, *e.g.*, for multiple sclerosis), proteolipid protein (PLP, *e.g.*, for multiple sclerosis), myelin-associated glycoprotein (MAG, *e.g.*, for anti-MAG peripheral neuropathy), insulin (*e.g.*, for type 1 diabetes), islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP, *e.g.*, for type 1 diabetes), preproinsulin (*e.g.*, for type 1 diabetes), glutamic decarboxylase (GAD, *e.g.*, for type 1 diabetes), tyrosine phosphatase like autoantigen (*e.g.*, for type 1 diabetes), insulinoma antigen- 2 (*e.g.*, for type 1 diabetes), islet cell antigen (*e.g.*, for type 1 diabetes); thyroid stimulating hormone (TSH) receptor (*e.g.*, for Graves' disease), thyrotropin receptor (*e.g.*, for Graves' disease), chondroitin sulfate proteoglycan 1 (*e.g.*, for rheumatoid arthritis), CD4+ T cell epitope (*e.g.*, GRVRVNSAY), *e.g.*, for proteoglycan induced arthritis (PGIA) or rheumatoid arthritis), or acetylcholine receptor (*e.g.*, for myasthenia gravis).

[0255] In certain embodiments, an autoimmune disease protein is a mammalian myelin oligodendrocyte glycoprotein (MOG), myelin basis protein (MBP), proteolipid protein (PLP), or a subsequence thereof. In some embodiments, an autoimmune disease protein is a human protein, such as human myelin basis protein (MBP), a human proteolipid protein (PLP), a human myelin oligodendrocyte glycoprotein (MOG), or a subsequence thereof.

[0256] Other heterologous nucleic acids encoding gene products useful in accordance with the instant invention include, for example and without limitation, reporters or detectable markers such as luciferase, green fluorescent protein (GFP), yellow fluorescent protein (YFP), blue fluorescent protein, cyan fluorescent protein, enhanced GFP, enhanced YFP, photoactivatable GFP, *Discosoma* species fluorescent protein (dsRed), mFruits, mCherry, TagRFPs, eqFP611, photoswitchable fluorescent proteins (for example Dronpa and EosFP), chloramphenicol acetyltransferase, Halo-tag fusion protein, alkaline phosphatase, horseradish peroxidase and beta-galactosidase.

[0257] In certain embodiments, heterologous nucleic acids comprise inhibitory DNA or encode inhibitory RNA (RNAi). Examples of inhibitory RNA include, for example and without limitation, small or short hairpin (sh)RNA, microRNA (miRNA), small or short interfering (si)RNA, trans-splicing RNA, and antisense RNA.

[0258] In certain embodiments, the heterologous nucleic acid encodes an inhibitory nucleic acid. In certain embodiments, the inhibitory nucleic acid is selected from the group consisting of a siRNA, an antisense molecule, miRNA, RNAi, a ribozyme and a shRNA. In certain embodiments, the inhibitory nucleic acid binds to a gene, a transcript of a gene, or a transcript of a gene associated with a polynucleotide repeat disease selected from the group consisting of a huntingtin (HTT) gene, a gene associated with dentatorubropallidoluysian atrophy (atrophin 1, ATN1), androgen receptor on the X chromosome in spinobulbar muscular atrophy, human Ataxin-1, -2, -3, and -7, $Ca_v2.1$ P/Q voltage-dependent calcium channel (CACNA1A), TATA-binding protein, Ataxin 8 opposite strand (ATXN8OS), Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B beta isoform in spinocerebellar ataxia (type 1, 2, 3, 6, 7, 8, 12 17), *FMR1* (fragile X mental retardation 1) in fragile X syndrome, *FMR1* (fragile X mental retardation 1) in fragile X-associated tremor/ataxia syndrome, *FMR1* (fragile X mental retardation 2) or AF4/FMR2 family member 2 in fragile XE mental retardation; Myotonin-protein kinase (MT-PK) in myotonic dystrophy; Frataxin in Friedreich's ataxia; a mutant of superoxide dismutase 1 (SOD1) gene in amyotrophic lateral sclerosis; a gene involved in pathogenesis of Parkinson's disease and/or Alzheimer's disease; apolipoprotein B (APOB) and proprotein convertase subtilisin/kexin type 9 (PCSK9), hypercholesterolemia; HIV Tat, human immunodeficiency virus transactivator of transcription gene, in HIV infection; HIV TAR, HIV TAR, human immunodeficiency virus transactivator response element gene, in HIV infection; C-C

chemokine receptor (CCR5) in HIV infection; Rous sarcoma virus (RSV) nucleocapsid protein in RSV infection, liver-specific microRNA (miR-122) in hepatitis C virus infection; p53, acute kidney injury or delayed graft function kidney transplant or kidney injury acute renal failure; protein kinase N3 (PKN3) in advance recurrent or metastatic solid malignancies; LMP2, LMP2 also known as proteasome subunit beta-type 9 (PSMB 9), metastatic melanoma; LMP7,also known as proteasome subunit beta-type 8 (PSMB 8), metastatic melanoma; MECL1 also known as proteasome subunit beta-type 10 (PSMB 10), metastatic melanoma; vascular endothelial growth factor (VEGF) in solid tumors; kinesin spindle protein in solid tumors, apoptosis suppressor B-cell CLL/lymphoma (BCL-2) in chronic myeloid leukemia; ribonucleotide reductase M2 (RRM2) in solid tumors; Furin in solid tumors; polo-like kinase 1 (PLK1) in liver tumors, diacylglycerol acyltransferase 1 (DGAT1) in hepatitis C infection, beta-catenin in familial adenomatous polyposis; beta2 adrenergic receptor, glaucoma; RTP801/Redd1 also known as DNA damage-inducible transcript 4 protein, in diabetic macular edema (DME) or age-related macular degeneration; vascular endothelial growth factor receptor I (VEGFR1) in age-related macular degeneration or choroidal neovascularization, caspase 2 in non-arteritic ischaemic optic neuropathy; keratin 6A N17K mutant protein in pachyonychia congenital; influenza A virus genome/gene sequences in influenza infection; severe acute respiratory syndrome (SARS) coronavirus genome/gene sequences in SARS infection; respiratory syncytial virus genome/gene sequences in respiratory syncytial virus infection; Ebola filovirus genome/gene sequence in Ebola infection; hepatitis B and C virus genome/gene sequences in hepatitis B and C infection; herpes simplex virus (HSV) genome/gene sequences in HSV infection, coxsackievirus B3 genome/gene sequences in coxsackievirus B3 infection; silencing of a pathogenic allele of a gene (allele-specific silencing) like torsin A (TOR1A) in primary dystonia, pan-class I and HLA-allele specific in transplant; and mutant rhodopsin gene (RHO) in autosomal dominantly inherited retinitis pigmentosa (adRP).

[0259] Nucleic acid molecules, vectors such as cloning, expression vectors (*e.g.*, vector genomes) and plasmids, may be prepared using recombinant DNA technology methods. The availability of nucleotide sequence information enables preparation of nucleic acid molecules by a variety of means. For example, a heterologous nucleic acid comprising a vector or plasmid can be made using various standard cloning, recombinant DNA technology, via cell expression or *in vitro* translation and chemical synthesis techniques. Purity of

polynucleotides can be determined through sequencing, gel electrophoresis and the like. For example, nucleic acids can be isolated using hybridization or computer-based database screening techniques. Such techniques include, but are not limited to: (1) hybridization of genomic DNA or cDNA libraries with probes to detect homologous nucleotide sequences; (2) antibody screening to detect polypeptides having shared structural features, for example, using an expression library; (3) polymerase chain reaction (PCR) on genomic DNA or cDNA using primers capable of annealing to a nucleic acid sequence of interest; (4) computer searches of sequence databases for related sequences; and (5) differential screening of a subtracted nucleic acid library.

[0260] Nucleic acids of the instant invention may be maintained as DNA in any convenient cloning vector. In certain embodiments, clones are maintained in a plasmid cloning/expression vector, such as pBluescript or pBluescript II (Stratagene, La Jolla, CA), which is propagated in a suitable *E. coli* host cell. Alternatively, nucleic acids may be maintained in a vector suitable for expression in mammalian cells..

[0261] Methods that are known in the art for generating rAAV virions include, for example, transfection using AAV vector and AAV helper sequences in conjunction with coinfection with one or more AAV helper virus(es) (*e.g.*, adenovirus, herpesvirus, or vaccinia virus) or transfection with a recombinant AAV vector, an AAV helper vector, and an accessory function vector. Methods for generating rAAV virions are described in, for example and without limitation, U.S. Pat. Nos. 6,001,650 and 6,004,797. Following recombinant rAAV vector production (*i.e.,* vector generation in cell culture systems), rAAV virions can be obtained from the host cells and cell culture supernatant and purified as set forth herein.

[0262] Methods to determine infectious titer of rAAV vector containing a transgene are known in the art (See, *e.g.*, Zhen *et al.*, (2004) Hum. Gene Ther. (2004) 15:709). Methods for assaying for empty capsids and AAV vector particles with packaged genomes are known (See, *e.g.*, Grimm *et al.*, Gene Therapy (1999) 6:1322-1330; Sommer *et al.*, Molec. Ther. (2003) 7:122-128).

[0263] To determine degraded/denatured capsid, purified rAAV can be subjected to SDS-polyacrylamide gel electrophoresis, consisting of any gel capable of separating the three capsid proteins, for example, a gradient gel, then running the gel until sample is separated,

and blotting the gel onto nylon or nitrocellulose membranes. Anti-AAV capsid antibodies are then used as primary antibodies that bind to denatured capsid proteins (See, *e.g.*, Wobus *et al.*, J. Virol. (2000) 74:9281-9293). A secondary antibody that binds to the primary antibody contains a means for detecting the primary antibody. Binding between the primary and secondary antibodies is detected semi-quantitatively to determine the amount of capsids.

[0264] rAAV vectors and other compositions, agents, drugs, biologics (proteins) can be incorporated into pharmaceutical compositions. Such pharmaceutical compositions are useful for, among other things, administration and delivery to a subject *in vivo* or *ex vivo*.

[0265] The term "isolated," when used as a modifier of a composition, means that the compositions are made by the hand of man or are separated, completely or at least in part, from their naturally occurring *in vivo* environment. Generally, isolated compositions are substantially free of one or more materials with which they normally associate with in nature, for example, one or more protein, nucleic acid, lipid, carbohydrate, cell membrane.

[0266] 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 a nucleic acid molecule. 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.

[0267] The term "isolated" does not exclude compositions herein or combinations produced by the hand of man, for example, a rAAV and/or a pharmaceutical formulation. The term "isolated" also does not exclude alternative physical forms of the composition, such as hybrids/chimeras, multimers/oligomers, modifications (*e.g.*, phosphorylation, glycosylation, lipidation) or derivatized forms, or forms expressed in host cells produced by the hand of man.

[0268] 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.). The preparation can comprise at least 75% by weight, or about 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).

[0269] 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 sequence. For example, when used in reference to a nucleic acid or 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.

[0270] In certain embodiments, pharmaceutical compositions also contain a pharmaceutically acceptable carrier or excipient. Such excipients include any pharmaceutical agent that does not itself induce an immune response harmful to the individual receiving the composition, and which may be administered without undue toxicity.

[0271] As used herein the term "pharmaceutically acceptable" and "physiologically acceptable" mean a biologically acceptable formulation, gaseous, liquid or solid, or mixture thereof, which is suitable for one or more routes of administration, *in vivo* delivery or contact. A "pharmaceutically acceptable" or "physiologically acceptable" composition is a material that is not biologically or otherwise undesirable, *e.g.*, the material may be administered to a subject without causing substantial undesirable biological effects. Thus, such a pharmaceutical composition may be used, for example in administering a nucleic acid, vector, viral particle or protein to a subject.

[0272] Pharmaceutically acceptable excipients include, but are not limited to, liquids such as water, saline, glycerol, sugars and ethanol. Pharmaceutically acceptable salts can also be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles.

[0273] The pharmaceutical composition may be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding, free base forms. In other cases, a preparation may be a lyophilized powder which may contain any or all of the following: 1-50 mM histidine, 0.1%-2% sucrose, and 2-7% mannitol, at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

[0274] Pharmaceutical compositions include solvents (aqueous or non-aqueous), solutions (aqueous or non-aqueous), emulsions (*e.g.*, oil-in-water or water-in-oil), suspensions, syrups, elixirs, dispersion and suspension media, coatings, isotonic and absorption promoting or delaying agents, compatible with pharmaceutical administration or *in vivo* contact or delivery. Aqueous and non-aqueous solvents, solutions and suspensions may include suspending agents and thickening agents. Such pharmaceutically acceptable carriers include tablets (coated or uncoated), capsules (hard or soft), microbeads, powder, granules and crystals. Supplementary active compounds (*e.g.*, preservatives, antibacterial, antiviral and antifungal agents) can also be incorporated into the compositions.

[0275] Pharmaceutical compositions can be formulated to be compatible with a particular route of administration or delivery, as set forth herein or known to one of skill in the art. Thus, pharmaceutical compositions include carriers, diluents, or excipients suitable for administration by various routes.

[0276] Compositions suitable for parenteral administration comprise aqueous and non-aqueous solutions, suspensions or emulsions of the active compound, which preparations are typically sterile and can be isotonic with the blood of the intended recipient. Illustrative examples include, for example and without limitation, water, buffered saline, Hanks' solution, Ringer's solution, dextrose, fructose, ethanol, animal, vegetable or synthetic oils. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as, for example and without limitation, sodium carboxymethyl cellulose, sorbitol, or dextran.

[0277] Additionally, suspensions of the active compounds may be prepared as appropriate oil injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

[0278] Cosolvents and adjuvants may be added to the formulation, examples of which include, for example and without limitation cosolvents containing hydroxyl groups or other polar groups, for example, alcohols, such as isopropyl alcohol; glycols, such as propylene glycol, polyethyleneglycol, polypropylene glycol, glycol ether; glycerol; polyoxyethylene alcohols and polyoxyethylene fatty acid esters. Examples of adjuvants include, for example

and without limitation, surfactants such as, soya lecithin and oleic acid; sorbitan esters such as sorbitan trioleate; and polyvinylpyrrolidone.

[0279] After pharmaceutical compositions have been prepared, they may be placed in an appropriate container and labeled for treatment. Such labeling could include amount, frequency, and method of administration.

[0280] Pharmaceutical compositions and delivery systems appropriate for the compositions, methods and uses of the instant invention are known in the art (*see, e.g.*, Remington: The Science and Practice of Pharmacy (2003) 20th ed., Mack Publishing Co., Easton, PA; Remington's Pharmaceutical Sciences (1990) 18th ed., Mack Publishing Co., Easton, PA; The Merck Index (1996) 12th ed., Merck Publishing Group, Whitehouse, NJ; Pharmaceutical Principles of Solid Dosage Forms (1993), Technonic Publishing Co., Inc., Lancaster, Pa.; Ansel and Stoklosa, Pharmaceutical Calculations (2001) 11th ed., Lippincott Williams & Wilkins, Baltimore, MD; and Poznansky *et al.*, Drug Delivery Systems (1980), R. L. Juliano, ed., Oxford, N.Y., pp. 253-315).

[0281] In certain embodiments, the nucleic acids, polynucleotides and expression cassettes of the instant invention are delivered or administered via AAV vector particles. In certain embodiments, the nucleic acids, polynucleotides and expression cassettes of the instant invention can be delivered or administered via other types of viral particles, including retroviral, adenoviral, helper-dependent adenoviral, hybrid adenoviral, herpes simplex virus, lentiviral, poxvirus, Epstein-Barr virus, vaccinia virus, and human cytomegalovirus particles.

[0282] In certain embodiments, the nucleic acids, polynucleotides and expression cassettes of the instant invention are delivered or administered with a non-viral delivery system. Non-viral delivery systems include for example, chemical methods, such as liposomes, nanoparticles, lipid nanoparticles, polymers, microparticles, microcapsules, micelles, or extracellular vesicles and physical methods, such as gene gun, electroporation, particle bombardment, ultrasound utilization and magnetofection.

[0283] In certain embodiments, the nucleic acids polynucleotides and expression cassettes of the instant invention are delivered as naked DNA, minicircles, transposons, or closed-ended linear duplex DNA.

[0284] In certain embodiments, the nucleic acids, polynucleotides and expression cassettes of the instant invention are delivered or administered in AAV vector particles, or other viral particles, that are further encapsulated or complexed with liposomes, nanoparticles, lipid nanoparticles, polymers, microparticles, microcapsules, micelles, or extracellular vesicles.

[0285] A "lipid nanoparticle" or "LNP" refers to a lipid-based vesicle useful for delivery of AAV and having dimensions on the nanoscale, *i.e.*, from about 10 nm to about 1000 nm, or from about 50 to about 500 nm, or from about 75 to about 127 nm. Without being bound by theory, the LNP is believed to provide the nucleic acid, polynucleotides, expression cassette, or AAV vector with partial or complete shielding from the immune system. Shielding allows delivery of the nucleic acid, polynucleotide, expression cassette, or AAV vector to a tissue or cell while avoiding inducing a substantial immune response against the nucleic acid, polynucleotide, expression cassette, or AAV vector *in vivo*. Shielding may also allow repeated administration without inducing a substantial immune response against the nucleic acid, polynucleotide, expression vector or AAV vector *in vivo* (*e.g.*, in a subject such as a human). Shielding may also improve or increase delivery efficiency *in vivo*.

[0286] The pI (isoelectric point) of AAV is in a range from about 6 to about 6.5. Thus, the AAV surface carries a slight negative charge. As such it may be beneficial for the LNP to comprise a cationic lipid such as, for example, an amino lipid. Exemplary amino lipids have been described in U.S. Patent Nos. 9,352,042, 9,220,683, 9,186,325, 9,139,554, 9,126,966 9,018,187, 8,999,351, 8,722,082, 8,642,076, 8,569,256, 8,466,122, and 7,745,651 and U.S. Patent Publication Nos. 2016/0213785, 2016/0199485, 2015/0265708, 2014/0288146, 2013/0123338, 2013/0116307, 2013/0064894, 2012/0172411, and 2010/0117125.

[0287] The terms "cationic lipid" and "amino lipid" are used interchangeably herein to include those lipids and salts thereof having one, two, three, or more fatty acid or fatty alkyl chains and a pH-titratable amino group (*e.g.*, an alkylamino or dialkylamino group). The cationic lipid is typically protonated (*i.e.*, positively charged) at a pH below the pKa of the cationic lipid and is substantially neutral at a pH above the pKa. The cationic lipids may also be titratable cationic lipids. In certain embodiments, the cationic lipids comprise: a protonatable tertiary amine (*e.g.*, pH-titratable) group; C18 alkyl chains, wherein each alkyl chain independently has 0 to 3 (*e.g.*, 0, 1, 2, or 3) double bonds; and ether, ester, or ketal linkages between the head group and alkyl chains.

[0288] Cationic lipids may include, without limitation, 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 1,2-di-γ-linolenyloxy-N,N-dimethylaminopropane (γ-DLenDMA), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA, also known as DLin-C2K-DMA, XTC2, and C2K), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), dilinoleylmethyl-3-dimethylaminopropionate (DLin-M-C2-DMA, also known as MC2), (6Z,9Z,28Z,31 Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (DLin-M-C3-DMA, also known as MC3), salts thereof, and mixtures thereof. Other cationic lipids also include, but are not limited to, 1,2-distearyloxy-N,N-dimethyl-3-aminopropane (DSDMA), 1,2-dioleyloxy-N,N-dimethyl-3-aminopropane (DODMA), 2,2-dilinoleyl-4-(3-dimethylaminopropyl)-[1,3]-dioxolane (DLin-K-C3-DMA), 2,2-dilinoleyl-4-(3-dimethylaminobutyl)-[1,3]-dioxolane (DLin-K-C4-DMA), DLen-C2K-DMA, γ-DLen-C2K-DMA, and (DLin-MP-DMA) (also known as 1-B11).

[0289] Still further cationic lipids may include, without limitation, 2,2-dilinoleyl-5-dimethylaminomethyl-[1,3]-dioxane (DLin-K6-DMA), 2,2-dilinoleyl-4-N-methylpepiazino-[1,3]-dioxolane (DLin-K-MPZ), 1,2-dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-dilinoleyoxy-3-(dimethylamino)acetoxypropane (DLin-DAC), 1,2-dilinoleyoxy-3-morpholinopropane (DLin-MA), 1,2-dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-linoleoyl-2-linoleyloxy-3-dimethylaminopropane (DLin-2-DMAP), 1,2-dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), 3-(N,N-dilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-dioleylamino)-1,2-propanedio (DOAP), 1,2-dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), 3-(N—(N′,N′-dimethylaminoethane)-carbamoyl)cholesterol (DC-Chol), N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), dioctadecylamidoglycyl spermine (DOGS), 3-dimethylamino-2-(cholest-5-en-3-beta-

oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA), 2-[5′-(cholest-5-en-3-beta-oxy)-3′-oxapentoxy)-3-dimethyl-1-(cis,cis-9′,1-2′-octadecadienoxy)propane (CpLinDMA), N,N-dimethyl-3,4-dioleyloxybenzylamine (DMOBA), 1,2-N,N′-dioleylcarbamyl-3-dimethylaminopropane (DOcarbDAP), 1,2-N,N′-dilinoleylcarbamyl-3-dimethylaminopropane (DLincarbDAP), dexamethasone-spermine (DS) and disubstituted spermine (D2S) or mixtures thereof.

[0290] A number of commercial preparations of cationic lipids can be used, such as, LIPOFECTIN® (including DOTMA and DOPE, available from GIBCO/BRL), and LIPOFECTAMINE® (comprising DOSPA and DOPE, available from GIBCO/BRL).

[0291] In certain embodiments, cationic lipid may be present in an amount from about 10% by weight of the LNP to about 85% by weight of the lipid nanoparticle, or from about 50 % by weight of the LNP to about 75% by weight of the LNP.

[0292] Sterols may confer fluidity to the LNP. As used herein, "sterol" refers to any naturally occurring sterol of plant (phytosterols) or animal (zoosterols) origin as well as non-naturally occurring synthetic sterols, all of which are characterized by the presence of a hydroxyl group at the 3-position of the steroid A-ring. The sterol can be any sterol conventionally used in the field of liposome, lipid vesicle or lipid particle preparation, most commonly cholesterol. Phytosterols may include campesterol, sitosterol, and stigmasterol. Sterols also includes sterol-modified lipids, such as those described in U.S. Patent Application Publication 2011/0177156. In certain embodiments, a sterol may be present in an amount from about 5% by weight of the LNP to about 50% by weight of the lipid nanoparticle or from about 10% by weight of the LNP to about 25% by weight of the LNP.

[0293] LNP can comprise a neutral lipid. Neutral lipids may comprise any lipid species which exists either in an uncharged or neutral zwitterionic form at physiological pH. Such lipids include, without limitation, diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, dihydrosphingomyelin, cephalin, and cerebrosides. The selection of neutral lipids is generally guided by consideration of, inter alia, particle size and the requisite stability. In certain embodiments, the neutral lipid component may be a lipid having two acyl groups (*e.g.*, diacylphosphatidylcholine and diacylphosphatidylethanolamine).

[0294] Lipids having a variety of acyl chain groups of varying chain length and degree of saturation are available or may be isolated or synthesized by well-known techniques. In certain embodiments, lipids containing saturated fatty acids with carbon chain lengths in the range of C14 to C22 may be used. In certain embodiments, lipids with mono or diunsaturated fatty acids with carbon chain lengths in the range of C14 to C22 are used. Additionally, lipids having mixtures of saturated and unsaturated fatty acid chains can be used. Exemplary neutral lipids include, without limitation, 1,2-dioleoyl-sn-glycero-3-phosphatidyl-ethanolamine (DOPE), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), or any related phosphatidylcholine. The neutral lipids may also be composed of sphingomyelin, dihydrosphingomyelin, or phospholipids with other head groups, such as serine and inositol.

[0295] In certain embodiments, the neutral lipid may be present in an amount from about 0.1% by weight of the lipid nanoparticle to about 75% by weight of the LNP, or from about 5% by weight of the LNP to about 15% by weight of the LNP.

[0296] LNP encapsulated nucleic acids, expression cassettes and AAV vector can be incorporated into pharmaceutical compositions, *e.g.*, a pharmaceutically acceptable carrier or excipient. Such pharmaceutical compositions are useful for, among other things, administration and delivery of LNP encapsulated acids, expression cassettes and AAV vector to a subject *in vivo* or *ex vivo*.

[0297] Preparations of LNP can be combined with additional components, which may include, for example and without limitation, polyethylene glycol (PEG) and sterols.

[0298] The term "PEG" refers to a polyethylene glycol, a linear, water-soluble polymer of ethylene PEG repeating units with two terminal hydroxyl groups. PEGs are classified by their molecular weights; for example, PEG 2000 has an average molecular weight of about 2,000 daltons, and PEG 5000 has an average molecular weight of about 5,000 daltons. PEGs are commercially available from Sigma Chemical Co. and other companies and include, for example and without limitation, the following functional PEGs: monomethoxypolyethylene glycol (MePEG-OH), monomethoxypolyethylene glycol-succinate (MePEG-S), monomethoxypolyethylene glycol-succinimidyl succinate (MePEG-S-NHS), monomethoxypolyethylene glycol-amine (MePEG-NH2), monomethoxypolyethylene glycol-

tresylate (MePEG-TRES), and monomethoxypolyethylene glycol-imidazolyl-carbonyl (MePEG-IM).

[0299] In certain embodiments, PEG may be a polyethylene glycol with an average molecular weight of about 550 to about 10,000 daltons and is optionally substituted by alkyl, alkoxy, acyl or aryl. In certain embodiments, the PEG may be substituted with methyl at the terminal hydroxyl position. In certain embodiments, the PEG may have an average molecular weight from about 750 to about 5,000 daltons, or from about 1,000 to about 5,000 daltons, or from about 1,500 to about 3,000 daltons or from about 2,000 daltons or of about 750 daltons. The PEG can be optionally substituted with alkyl, alkoxy, acyl or aryl. In certain embodiments, the terminal hydroxyl group may be substituted with a methoxy or methyl group.

[0300] PEG-modified lipids include, for example and without limitation, the PEG-dialkyloxypropyl conjugates (PEG-DAA) described in U.S. Patent Nos. 8,936,942 and 7,803,397. PEG-modified lipids (or lipid-polyoxyethylene conjugates) that are useful may have a variety of "anchoring" lipid portions to secure the PEG portion to the surface of the lipid vesicle. Examples of suitable PEG-modified lipids include, for example and without limitation, PEG-modified phosphatidylethanolamine and phosphatidic acid, PEG-ceramide conjugates (*e.g.*, PEG-CerC14 or PEG-CerC20) which are described in U.S. Patent No. 5,820,873, PEG-modified dialkylamines and PEG-modified 1,2-diacyloxypropan-3-amines. In certain embodiments, the PEG-modified lipid may be PEG-modified diacylglycerols and dialkylglycerols. In certain embodiments, the PEG may be in an amount from about 0.5% by weight of the LNP to about 20% by weight of the LNP, or from about 5% by weight of the LNP to about 15% by weight of the LNP.

[0301] Furthermore, LNP can be a PEG-modified and a sterol-modified LNP. The LNPs, combined with additional components, can be the same or separate LNPs. In other words, the same LNP can be PEG modified and sterol modified or, alternatively, a first LNP can be PEG modified and a second LNP can be sterol modified. Optionally, the first and second modified LNPs can be combined.

[0302] In certain embodiments, prior to encapsulating LNPs may have a size in a range from about 10 nm to 500 nm, or from about 50 nm to about 200 nm, or from 75 nm to about

125 nm. In certain embodiments, LNP encapsulated nucleic acid, expression vector or AAV vector may have a size in a range from about 10 nm to 500 nm.

[0303] An "effective amount" or "sufficient amount" refers to an amount that provides, in single or multiple doses, alone or in combination, with one or more other compositions (therapeutic or immunosuppressive agents such as a drug like prednisone), treatments, protocols, or therapeutic regimens agents, a detectable response of any duration of time (long or short term), an expected or desired outcome in or a benefit to a subject of any measurable or detectable degree or for any duration of time (*e.g.*, for minutes, hours, days, months, years, or cured).

[0304] Doses can vary and depend upon the type, onset, progression, severity, frequency, duration, or probability of the disease to which treatment is directed, the clinical endpoint desired, previous or simultaneous treatments, the general health, age, gender, race or immunological competency of the subject and other factors that will be appreciated by the skilled artisan. The dose amount, number, frequency or duration may be proportionally increased or reduced, as indicated by any adverse side effects, complications or other risk factors of the treatment or therapy and the status of the subject. The skilled artisan will appreciate the factors that may influence the dosage and timing required to provide an amount sufficient for providing a therapeutic or prophylactic benefit.

[0305] The dose to achieve a therapeutic effect, *e.g.*, the dose in vector genomes/per kilogram of body weight (vg/kg), will vary based on several factors including, but not limited to: route of administration, the level of heterologous polynucleotide expression required to achieve a therapeutic effect, the specific disease treated, any host immune response to the viral vector, a host immune response to the heterologous polynucleotide or expression product (protein), and the stability of the protein expressed. One skilled in the art can determine a rAAV/vector genome dose range to treat a patient having a particular disease or disorder based on the aforementioned factors, as well as other factors.

[0306] Generally, doses will range from at least 1×10^{8}, or more, for example, 1×10^{9}, 1×10^{10}, 1×10^{11}, 1×10^{12}, 1×10^{13} or 1×10^{14}, or more, vector genomes per kilogram (vg/kg) of the weight of the subject, to achieve a therapeutic effect. AAV dose in the range of 1×10^{10}-1×10^{11} vg/kg in mice, and 1×10^{12}-1×10^{13} vg/kg in dogs have been effective. Doses can be

less, for example, a dose of less than 6×10^{12} vector genomes per kilogram (vg/kg). More particularly, a dose from about $1x10^{11}$ vg/kg to about $5x10^{12}$ vg/kg, or from about $5x10^{11}$ vg/kg to about $2x10^{12}$ vg/kg, or from about $5x10^{11}$ vg/kg to about $1x10^{12}$ vg/kg.

[0307] For Pompe disease, an effective amount would be an amount of GAA that inhibits or reduces glycogen production or accumulation, enhances or increases glycogen degradation or removal, reduces lysosomal alterations in tissues of the body of a subject, or improves muscle tone and/or muscle strength and/or respiratory function in a subject, for example. Effective amounts can be determined, for example, by ascertaining the kinetics of GAA uptake by myoblasts from plasma. Myoblasts GAA uptake rates (*K uptake*) of about 141 – 147 nM may appear to be effective (see, *e.g.*, Maga *et al.*, J. Biol. Chem. 2012) In animal models, GAA activity levels in plasma of greater than about 1,000 nmol/hr/mL, for example, about 1,000 to about 2,000 nmol/hr/mL have been observed to be therapeutically effective.

[0308] Using hemophilia B as an example, generally speaking, it is believed that, in order to achieve a therapeutic effect, a blood coagulation factor concentration that is greater than 1% of factor concentration found in a normal individual is needed to change a severe disease phenotype to a moderate one. A severe phenotype is characterized by joint damage and life-threatening bleeds. To convert a moderate disease phenotype into a mild one, it is believed that a blood coagulation factor concentration greater than 5% of normal is needed.

[0309] Diagnosis and disease severity classification for hemophilia A and B are based on the results of factor VIII and factor IX activity assays, respectively. The two main assays used to assess factor activity are one-stage assays (OSAs), based on activated partial thromboplastin time (aPTT), and two-stage chromogenic substrate assays (CSAs), which use a factor Xa-based enzymatic chromophore substrate reaction. Such assays are well known in the art and are further described in Adcock *et al.*, 2018, Int. J. Lab. Hem., 40:621-629.

[0310] FVIII levels in normal humans are about 150-200 ng/mL plasma, but may be less (*e.g.*, range of about 100-150 ng/mL) or greater (*e.g.*, range of about 200-300 ng/mL) and still considered normal, due to functional clotting as determined, for example, by an aPTT one-stage clotting assay. Thus, a therapeutic effect can be achieved by expression of FVIII or hFVIII-BDD such that the total amount of FVIII in the subject/human is greater than 1% of the FVIII present in normal subjects/humans, *e.g.*, 1% of 100-300 ng/mL.

[0311] rAAV vector doses can be at a level, typically at the lower end of the dose spectrum, such that there is not a substantial immune response against the FVIII or AAV vector. More particularly, a dose of up to but less than $6x10^{12}$ vg/kg, such as about $5x10^{11}$ to about $5x10^{12}$ vg/kg, or more particularly, about $5x10^{11}$ vg/kg or about $1x10^{12}$ vg/kg.

[0312] In certain embodiments, the rAAV vector dose is at a level to deliver a safe and effective amount of FVIII and provide therapeutic benefit to a subject with hemophilia A with inhibitory antibodies against FVIII (hemophilia A with inhibitors).

[0313] The doses of an "effective amount" or "sufficient amount" for treatment (*e.g.*, to ameliorate or to provide a therapeutic benefit or improvement) typically are effective to provide a response to one, multiple or all adverse symptoms, consequences or complications of the disease, one or more adverse symptoms, disorders, illnesses, pathologies, or complications, for example, caused by or associated with the disease, to a measurable extent, although decreasing, reducing, inhibiting, suppressing, limiting or controlling progression or worsening of the disease is a satisfactory outcome.

[0314] An effective amount or a sufficient amount can but need not be provided in a single administration, may require multiple administrations, and, can but need not be, administered alone or in combination with another composition (*e.g.*, agent), treatment, protocol or therapeutic regimen. For example, the amount may be proportionally increased as indicated by the need of the subject, type, status and severity of the disease treated or side effects (if any) of treatment. In addition, an effective amount or a sufficient amount need not be effective or sufficient if given in single or multiple doses without a second composition (*e.g.*, another drug or agent), treatment, protocol or therapeutic regimen, since additional doses, amounts or duration above and beyond such doses, or additional compositions (*e.g.*, drugs or agents), treatments, protocols or therapeutic regimens may be included in order to be considered effective or sufficient in a given subject. Amounts considered effective also include amounts that result in a reduction of the use of another treatment, therapeutic regimen or protocol, such as administration of recombinant clotting factor protein (*e.g.*, FVIII) for treatment of a clotting disorder (*e.g.*, hemophilia A or hemophilia A with inhibitory antibodies against FVIII, also known as hemophila A with inhibitors).

[0315] Accordingly, methods and uses of the instant invention also include, among other things, methods and uses that result in a reduced need or use of another compound, agent,

drug, therapeutic regimen, treatment protocol, process, or remedy. For example, for a blood clotting disease, a method or use of the instant invention has a therapeutic benefit if in a given subject a less frequent or reduced dose or elimination of administration of a recombinant clotting factor protein to supplement for the deficient or defective (abnormal or mutant) endogenous clotting factor in the subject. Thus, in accordance with the instant invention, methods and uses of reducing need or use of another treatment or therapy are provided.

[0316] An effective amount or a sufficient amount need not be effective in each and every subject treated, nor a majority of treated subjects in a given group or population. An effective amount or a sufficient amount means effectiveness or sufficiency in a particular subject, not a group or the general population. As is typical for such methods, some subjects will exhibit a greater response, or less or no response to a given treatment method or use.

[0317] The term "ameliorate" means a detectable or measurable improvement in a subject's disease or symptom thereof, or an underlying cellular response. A detectable or measurable improvement includes a subjective or objective decrease, reduction, inhibition, suppression, limit or control in the occurrence, frequency, severity, progression, or duration of the disease, or complication caused by or associated with the disease, or an improvement in a symptom or an underlying cause or a consequence of the disease, or a reversal of the disease. For HemA, an effective amount would be an amount that reduces frequency or severity of acute bleeding episodes in a subject, for example, or an amount that reduces clotting time as measured by a clotting assay, for example.

[0318] Accordingly, pharmaceutical compositions of the instant invention include compositions wherein the active ingredients are contained in an effective amount to achieve the intended therapeutic purpose. Determining a therapeutically effective dose is well within the capability of a skilled medical practitioner using the techniques and guidance provided in the instant invention.

[0319] Therapeutic doses will depend on, among other factors, the age and general condition of the subject, the severity of the aberrant phenotype, and the strength of the control sequences regulating expression levels. Thus, a therapeutically effective amount in humans will fall in a relatively broad range that may be determined by a medical practitioner based on the response of an individual patient to a vector-based treatment. Such doses may be alone or in combination with an immunosuppressive agent or drug.

[0320] Compositions such as pharmaceutical compositions may be delivered to a subject, so as to allow transgene expression and optionally production of encoded protein. In certain embodiments, pharmaceutical compositions comprising sufficient genetic material to enable a recipient to produce a therapeutically effective amount of a blood-clotting factor to influence hemostasis in the subject.

[0321] The compositions may be administered alone. In certain embodiments, a recombinant AAV particle provides a therapeutic effect without an immunosuppressive agent. The therapeutic effect optionally is sustained for a period of time, *e.g.*, 2-4, 4-6, 6-8, 8-10, 10-14, 14-20, 20-25, 25-30, or 30-50 days or more, for example, 50-75, 75-100, 100-150, 150-200 days or more without administering an immunosuppressive agent. Accordingly, in certain embodiments rAAV virus particle provides a therapeutic effect without administering an immunosuppressive agent for a period of time.

[0322] The compositions of the instant inventon may be administered in combination with at least one other inert or therapeutic agent. In certain embodiments, rAAV vector is administered in conjunction with one or more immunosuppressive agents prior to, substantially at the same time or after administering a rAAV vector. In certain embodiments, *e.g.*, 1-12, 12-24 or 24-48 hours, or 2-4, 4-6, 6-8, 8-10, 10-14, 14-20, 20-25, 25-30, 30-50, or more than 50 days following administering rAAV vector. Such administration of immunosuppressive agents after a period of time following administering rAAV vector if there is a decrease in encoded protein expression after the initial expression levels for a period of time, *e.g.*, 20-25, 25-30, 30-50, 50-75, 75-100, 100-150, 150-200 or more than 200 days following rAAV vector.

[0323] In certain embodiments, an immunosuppressive agent is an anti-inflammatory agent. In certain embodiments, an immunosuppressive agent is a steroid. In certain embodiments, an immunosuppressive agent is prednisone, cyclosporine (*e.g.*, cyclosporine A), mycophenolate, rituximab, rapamycin, or a derivative thereof. In certain embodiments, agents include a stabilizing compound. Other immunosuppressive agents that can be used according to the instant invention include, for example and without limitation, a B cell targeting antibody, *e.g.*, rituximab; a proteasome inhibitor, *e.g.*, bortezomib; a mammalian target of rapamycin (mTOR) inhibitor, *e.g.*, rapamycin; a tyrosine kinase inhibitor, *e.g.*,

ibrutinib; an inhibitor of B-cell activating factor (BAFF); and an inhibitor of a proliferation-inducing ligand (APRIL).

[0324] Compositions may be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The compositions may be administered to a patient alone, or in combination with other agents (*e.g.*, co-factors) which influence hemostasis.

[0325] Methods and uses of the instant invention include delivery and administration systemically, regionally or locally, or by any route, for example and without limitation, by injection or infusion. Delivery of the pharmaceutical compositions *in vivo* may generally be accomplished via injection using a conventional syringe, although other delivery methods such as convection-enhanced delivery are envisioned (See *e.g.*, U.S. Pat. No. 5,720,720). For example, compositions may be delivered subcutaneously, epidermally, intradermally, intrathecally, intraorbitally, intramucosally, intraperitoneally, intravenously, intra-pleurally, intraarterially, orally, intrahepatically, via the portal vein, or intramuscularly. Other modes of administration include oral and pulmonary administration, suppositories, and transdermal applications. A clinician specializing in the treatment of patients with blood coagulation or clotting factor disorders, for example, may determine the optimal route for administration of the adenoviral-associated vectors based on a number of criteria, including, but not limited to: the condition of the patient and the purpose of the treatment (*e.g.*, increased GAA, enhanced or reduced blood coagulation, etc.).

[0326] Methods of treatment according to the instant nvention include combination therapies that include the additional use of one or more of any compound, agent, drug, treatment or other therapeutic regimen or protocol having a desired therapeutic, beneficial, additive, synergistic or complementary activity or effect. Exemplary combination compositions and treatments include, for example and without limitation, second actives, such as, biologics (proteins), agents (*e.g.*, immunosuppressive agents) and drugs. Such biologics (proteins), agents, drugs, treatments and therapies can be administered or performed prior to, substantially contemporaneously with or following any other method of treatment according to the instant invention, for example, a therapeutic method of treating a subject for a lysosomal storage disease such as Pompe, or a therapeutic method of treating a subject for a blood clotting disease such as HemA or HemB.

[0327] The compound, agent, drug, treatment or other therapeutic regimen or protocol can be administered as a combination composition, or administered separately, such as concurrently or in series or sequentially (prior to or following) delivery or administration of a nucleic acid, vector, recombinant vector (*e.g.*, rAAV), or recombinant virus particle. The instant invention therefore provides combinations in which a method of treatment according to the instant invention is in a combination with any compound, agent, drug, therapeutic regimen, treatment protocol, process, remedy or composition, set forth herein or known to one of skill in the art. The compound, agent, drug, therapeutic regimen, treatment protocol, process, remedy or composition can be administered or performed prior to, substantially contemporaneously with or following administration of a nucleic acid, vector, recombinant vector (*e.g.*, rAAV), or recombinant virus particle administered to a patient or subject according to the instant invention.

[0328] The instant invention may be used in human and veterinary medical applications. Suitable subjects therefore include mammals, such as humans, as well as non-human mammals. The term "subject" refers to an animal, typically a mammal, such as humans, non-human primates (apes, gibbons, gorillas, chimpanzees, orangutans, macaques), a domestic animal (dogs and cats), a farm animal (poultry such as chickens and ducks, horses, cows, goats, sheep, pigs), and experimental animals (mouse, rat, rabbit, guinea pig). Human subjects include fetal, neonatal, infant, juvenile and adult subjects. Subjects include animal disease models, for example, mouse and other animal models of blood clotting diseases such as HemA and others known to those of skill in the art.

[0329] Subjects appropriate for treatment in accordance with the instant invention include those having or at risk of producing an insufficient amount or having a deficiency in a functional gene product (*e.g.*, GAA or a blood clotting factor, such as FVIII or FIX), or produce an aberrant, partially functional or non-functional gene product (*e.g.*, GAA or a blood clotting factor such as FVIII or FIX), which can lead to disease. Subjects appropriate for treatment in accordance with the instant invention also include those having or at risk of producing an aberrant, or defective (mutant) gene product (protein) that leads to a disease such that reducing amounts, expression or function of the aberrant, or defective (mutant) gene product (protein) would lead to treatment of the disease, or reduce one or more symptoms or ameliorate the disease. Target subjects, for example, include subjects having aberrant, insufficient or absent blood clotting factor production, such as hemophiliacs (*e.g.*, hemophilia

A or hemophilia B), or subjects having aberrant, insufficient or absent GAA production, such as subjects with Pompe disease.

[0330] Subjects include those with no detectable neutralizing antibodies against AAV. Subjects also include those with neutralizing antibodies against AAV. Such subjects may have low titer neutralizing antibodies against AAV.

[0331] Subjects can be tested for an immune response, *e.g.*, antibodies against AAV. Candidate subjects (for example, hemophilia or Pompe disease subjects) can be screened prior to treatment according to a method of the instant invention. Subjects also can be tested for antibodies against AAV after treatment, and optionally monitored for a period of time after treatment. Subjects developing antibodies can be treated with an immunosuppressive agent (*e.g.*, prednisone), or can be administered one or more additional amounts of AAV vector.

[0332] Subjects considered negative for antibodies that bind to AAV have a titer of less than 1:1. Subjects that have antibodies that bind to AAV can have a titer of greater than 1:1 but less than 1:5. Subjects may also have the same or greater than 1:5 AAV antibody titer. These antibody titers can be calculated, for example, by performing serial dilutions of a blood, plasma or serum (or other body fluid) sample from a subject, and the first dilution at which the sample inhibits AAV transduction by 50% or more, as measured by reporter activity in an *in vitro* cell-based assay, is reported as the antibody titer.

[0333] Strategies to reduce (overcome) or avoid humoral immunity to AAV in systemic gene transfer include, administering high vector doses, use of AAV empty capsids as decoys to adsorb anti-AAV antibodies, administration of immunosuppressive drugs to decrease, reduce, inhibit, prevent or eradicate the humoral immune response to AAV, changing the AAV capsid serotype or engineering the AAV capsid to be less susceptible to neutralizing antibodies, use of plasma exchange cycles to adsorb anti-AAV immunoglobulins, thereby reducing anti-AAV antibody titer, use of delivery techniques such as balloon catheters followed by saline flushing (Mingozzi *et al.*, 2013, Blood, 122:23-36), and immunoadsorption (US patent application publication US 2018/0169273 A1).

[0334] Subjects appropriate for treatment in accordance with the instant invention also include those having or at risk of producing antibodies against AAV. rAAV vectors can be

administered or delivered to such subjects using several techniques. For example, empty capsid AAV (*i.e.*, AAV lacking a transgene) can be delivered to bind to the AAV antibodies in the subject thereby allowing the AAV vector bearing nucleic acid or nucleic acid variant to transform cells of the subject.

[0335] Ratio of empty capsids to the rAAV vector can be between about 2:1 to about 50:1, or between about 2:1 to about 25:1, or between about 2:1 to about 20:1, or between about 2:1 to about 15:1, or between about 2:1 to about 10:1. Ratios can also be about 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, or 10:1.

[0336] Amounts of empty capsid AAV to administer can be calibrated based upon the amount (titer) of AAV antibodies produced in a particular subject. Empty capsid can be of any AAV serotype, for example, AAV1, AAV2, AAV3, AAV3B, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, Rh10, Rh74, AAV-2i8, LK03 (SEQ ID NO:91), SPK (SEQ ID NO:92).

[0337] Alternatively, or in addition to, AAV vector can be delivered by direct intramuscular injection (*e.g.*, one or more slow-twitch fibers of a muscle). In another alternative, a catheter introduced into the femoral artery can be used to delivery AAV vectors to liver via the hepatic artery. Non-surgical means can also be employed, such as endoscopic retrograde cholangiopancreatography (ERCP), to deliver AAV vectors directly to the liver, thereby bypassing the bloodstream and AAV antibodies. Other ductal systems, such as the ducts of the submandibular gland, can also be used as portals for delivering AAV vectors into a subject that develops or has preexisting anti-AAV antibodies.

[0338] Administration or *in vivo* delivery to a subject can be performed prior to development of an adverse symptom, condition, complication, etc. caused by or associated with the disease. For example, a screen (*e.g.*, genetic) can be used to identify such subjects as candidates for invention compositions, methods and uses. Such subjects therefore include those screened positive for an insufficient amount or a deficiency in a functional gene product (*e.g.*, blood clotting factor), or that produce an aberrant, partially functional or non-functional gene product (*e.g.*, blood clotting factor).

[0339] Administration or *in vivo* delivery to a subject in accordance with the methods and uses of the instant invention as disclosed herein can be practiced within 1-2, 2-4, 4-12, 12-24

or 24-72 hours after a subject has been identified as having the disease targeted for treatment, has one or more symptoms of the disease, or has been screened and is identified as positive as set forth herein even though the subject does not have one or more symptoms of the disease. Of course, methods and uses of the instant invention can be practiced 1-7, 7-14, 14-21, 21-48 or more days, months or years after a subject has been identified as having the disease targeted for treatment, has one or more symptoms of the disease, or has been screened and is identified as positive as set forth herein.

[0340] A "unit dosage form" as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity optionally in association with a pharmaceutical carrier (excipient, diluent, vehicle or filling agent) which, when administered in one or more doses, is calculated to produce a desired effect (*e.g.*, prophylactic or therapeutic effect). Unit dosage forms may be within, for example, ampules and vials, which may include a liquid composition, or a composition in a freeze-dried or lyophilized state; a sterile liquid carrier, for example, can be added prior to administration or delivery *in vivo*. Individual unit dosage forms can be included in multi-dose kits or containers. Recombinant vector (*e.g.*, rAAV) sequences, recombinant virus particles, and pharmaceutical compositions thereof can be packaged in single or multiple unit dosage form for ease of administration and uniformity of dosage.

[0341] Subjects can be tested for protein or activity levels of a relevant gene product (*e.g.*, GAA or a blood clotting factor, such as FVIII or FIX) to determine if such subjects are appropriate for treatment according to a method of the instant invention. For example, candidate hemophilia A subjects can be tested for FVIII amounts or activity prior to treatment according to a method of the instant invention; candidate Pompe subjects can be tested for GAA amounts or activity prior to treatment according to the instant invention. Subjects also can be tested for amounts of FVIII or GAA protein or activity after treatment according to a method of the instant invention. Such treated subjects can be monitored after treatment for blood clotting activity (for HemA) or for GAA activity (for Pompe), periodically, *e.g.*, every 1-4 weeks, 1-6 months, or 1, 2, 3, 4, 5 or more years.

[0342] Subjects can be tested for one or more liver enzymes for an adverse response or to determine if such subjects are appropriate for treatment according to a method of the instant invention. For examples, candidate hemophilia or Pompe subjects can be screened for

amounts of one or more liver enzymes prior to treatment according to a method of the instant invention. Subjects also can be tested for amounts of one or more liver enzymes after treatment according to a method of the instant invention. Such treated subjects can be monitored after treatment for elevated liver enzymes, periodically, *e.g.*, every 1-4 weeks or 1-6 months.

[0343] Exemplary liver enzymes include alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH), but other enzymes indicative of liver damage can also be monitored. A normal level of these enzymes in the circulation is typically defined as a range that has an upper level, above which the enzyme level is considered elevated, and therefore indicative of liver damage. A normal range depends in part on the standards used by the clinical laboratory conducting the assay.

[0344] In certain embodiments, subjects with bleeding disorders can be monitored for bleeding episodes to determine if such subjects are eligible for or responding to treatment according to the instant invention, and/or the amount or duration of responsiveness. Subjects can be monitored for bleeding episodes to determine if such subjects are in need of an additional treatment, *e.g.*, a subsequent AAV vector administration or administration of an immunosuppressive agent, or more frequent monitoring. Hemophilia subjects can be monitored for bleeding episodes prior to and after treatment according to a method of the instant invention. Subjects also can be tested for frequency and severity of bleeding episodes during or after treatment according to a method of the instant invention.

[0345] In certain embodiments subjects with Pompe disease or in need of GAA can be monitored by a variety of tests, assays and functional assessments to demonstrate, measure and/or assess efficacy of GAA, to determine if such subjects are eligible for or responding to treatment, or are in need of additional treatment, in accordance with the instant invention.

[0346] The instant invention provides kits with packaging material and one or more components therein. A kit typically includes a label or packaging insert including a description of the components or instructions for use *in vitro*, *in vivo*, or *ex vivo*, of the components therein. A kit can contain a collection of such components, *e.g.*, a nucleic acid, recombinant vector, virus (*e.g.*, AAV) vector, or virus particle and optionally a second active, such as another compound, agent, drug or composition.

[0347] A kit refers to a physical structure housing one or more components of the kit. Packaging material can maintain the components sterilely, and can be made of material commonly used for such purposes (*e.g.*, paper, corrugated fiber, glass, plastic, foil, ampules, vials, tubes, etc.).

[0348] Labels or inserts can include identifying information of one or more components therein, dose amounts, clinical pharmacology of the active ingredient(s) including mechanism of action, pharmacokinetics and pharmacodynamics. Labels or inserts can include information identifying manufacturer, lot numbers, manufacture location and date, expiration dates. Labels or inserts can include information identifying manufacturer information, lot numbers, manufacturer location and date. Labels or inserts can include information on a disease for which a kit component may be used. Labels or inserts can include instructions for the clinician or subject for using one or more of the kit components in a method, use, or treatment protocol or therapeutic regimen. Instructions can include dosage amounts, frequency or duration, and instructions for practicing any of the methods, uses, treatment protocols or prophylactic or therapeutic regimes described herein.

[0349] Labels or inserts can include information on any benefit that a component may provide, such as a prophylactic or therapeutic benefit. Labels or inserts can include information on potential adverse side effects, complications or reactions, such as warnings to the subject or clinician regarding situations where it would not be appropriate to use a particular composition. Adverse side effects or complications could also occur when the subject has, will be or is currently taking one or more other medications that may be incompatible with the composition, or the subject has, will be or is currently undergoing another treatment protocol or therapeutic regimen which would be incompatible with the composition and, therefore, instructions could include information regarding such incompatibilities.

[0350] Labels or inserts include "printed matter," *e.g.*, paper or cardboard, or separate or affixed to a component, a kit or packing material (*e.g.*, a box), or attached to an ampule, tube or vial containing a kit component. Labels or inserts can additionally include a computer readable medium, such as a bar-coded printed label, a disk, optical disk such as CD- or DVD-ROM/RAM, DVD, MP3, magnetic tape, or an electrical storage media such as RAM and

ROM or hybrids of these such as magnetic/optical storage media, FLASH media or memory type cards.

[0351] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the instant invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described herein.

[0352] All patents, patent applications, publications, and other references, GenBank citations and ATCC citations cited herein are incorporated by reference in their entirety. In case of conflict, the specification, including definitions, will control.

[0353] Various terms relating to the biological molecules of the instant invention are used hereinabove and also throughout the specification and claims.

[0354] All of the features disclosed herein may be combined in any combination. Each feature disclosed in the specification may be replaced by an alternative feature serving a same, equivalent, or similar purpose. Thus, unless expressly stated otherwise, disclosed features (*e.g.*, CpG reduced) nucleic acids, vectors, plasmids, expression/recombinant vectors (*e.g.*, rAAV) sequences, or recombinant virus particles) are an example of a genus of equivalent or similar features.

[0355] As used herein, the singular forms "a", "and," and "the" include plural referents unless the context clearly indicates otherwise. Thus, for example, reference to "a nucleic acid" includes a plurality of such nucleic acids, reference to "a vector" includes a plurality of such vectors, and reference to "a virus" or "particle" includes a plurality of such viruses/particles.

[0356] As used herein, all numerical values or numerical ranges include integers within such ranges and fractions of the values or the integers within ranges unless the context clearly indicates otherwise. Thus, to illustrate, reference to 80% or more identity, includes 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, etc., as well as 81.1%, 81.2%, 81.3%, 81.4%, 81.5%, etc., 82.1%, 82.2%, 82.3%, 82.4%, 82.5%, etc., and so forth.

[0357] Reference to an integer with more (greater) or less than includes any number greater or less than the reference number, respectively. Thus, for example, a reference to less than 100, includes 99, 98, 97, etc. all the way down to the number one (1); and less than 10, includes 9, 8, 7, etc. all the way down to the number one (1).

[0358] As used herein, all numerical values or ranges include fractions of the values and integers within such ranges and fractions of the integers within such ranges unless the context clearly indicates otherwise. Thus, to illustrate, reference to a numerical range, such as 1-10 includes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, as well as 1.1, 1.2, 1.3, 1.4, 1.5, etc., and so forth. Reference to a range of 1-50 therefore includes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, etc., up to and including 50, as well as 1.1, 1.2, 1.3, 1.4, 1.5, etc., 2.1, 2.2, 2.3, 2.4, 2.5, etc., and so forth.

[0359] Reference to a series of ranges includes ranges which combine the values of the boundaries of different ranges within the series. Thus, to illustrate reference to a series of ranges, for example, of 1-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-75, 75-100, 100-150, 150-200, 200-250, 250-300, 300-400, 400-500, 500-750, 750-850, includes ranges of 1-20, 1-30, 1-40, 1-50, 1-60, 10-30, 10-40, 10-50, 10-60, 10-70, 10-80, 20-40, 20-50, 20-60, 20-70, 20-80, 20-90, 50-75, 50-100, 50-150, 50-200, 50-250, 100-200, 100-250, 100-300, 100-350, 100-400, 100-500, 150-250, 150-300, 150-350, 150-400, 150-450, 150-500, etc.

[0360] The instant invention is generally disclosed herein using affirmative language to describe the numerous embodiments of the instant invention. The instant invention also specifically includes embodiments in which particular subject matter is excluded, in full or in part, such as substances or materials, method steps and conditions, protocols, or procedures. For example, in certain embodiments of the instant invention, materials and/or method steps are excluded. Thus, even though the instant invention is generally not expressed herein in terms of what the instant invention does not include aspects that are not expressly excluded in the instant invention are nevertheless disclosed herein.

[0361] A number of embodiments of the instant invention have been described. Nevertheless, one skilled in the art, without departing from the spirit and scope of the instant invention, can make various changes and modifications of the instant invention to adapt it to various usages and conditions. Accordingly, the following examples are intended to illustrate but not limit the scope of the instant invention claimed in any way.

Examples

EXAMPLE 1: Methods

[0362] hFVIII ELISA of NHP plasma: 96-well plates were coated with human-specific FVIII antibody overnight, washed, and blocked prior to incubation with diluted NHP study subject plasma samples. Standard curves were generated by incubating additional wells with a dilution series of recombinant B-domain deleted hFVIII, Xyntha® (Pfizer). Plates were washed and subsequently incubated with biotinylated human-specific FVIII detection antibody. Plates were incubated with horseradish peroxidase (HRP) conjugated streptavidin, treated with TMB substrate, and read on a microplate reader to determine absorbance at 450 nm.

[0363] hFVIII ELISA of mouse plasma: hFVIII ELISA of mouse plasma was performed essentially as described above for NHPs; however, the capture and detection antibodies utilized differed.

[0364] Cell-based assay to measure the potency of AAV vectors encoding human FVIII transgenes: Huh7 cells were plated in 48-well dishes at 5 x 10^4 cells per well overnight. Post study, remaining undiluted stock vector and vector diluted for dosing were prepared in a 10-fold dose curve (MOI ranging from 1 x 10^6 - 1 x 10^3) in DMEM +10% FBS + penicillin/streptomycin/L-glutamine. Existing medium was removed from Huh7 cells and replaced with virus particle containing medium. Cells were maintained at 37 °C and 5% CO_2 for 72 hours and supernatants were harvested and stored in low retention microtiter plates at -80 °C until assayed for hFVIII activity. Supernatants were assayed by Coatest® SP4 Factor VIII (Chromogenix) with a standard curve generated by diluting recombinant B-domain deleted hFVIII, Xyntha® (Pfizer), into cell growth medium.

[0365] Cell-based assay to measure the protein expression efficiency of plasmids encoding human FVIII transgenes: Huh7 cells were plated in 48-well dishes at 5 x 10^4 cells per well overnight in DMEM +10% FBS + penicillin/streptomycin/L-glutamine. Plasmids were prepared using Plasmid Giga Kit (Qiagen) and transfected into cells at 250 ng per well using Polyethylenimine (PEI) Max. Cells were maintained at 37 °C and 5% CO_2 for 72 hours and supernatants were harvested and stored in low retention microtiter plates at -80 °C until assayed for hFVIII activity. Supernatants were assayed by Coatest® SP4 Factor VIII

(Chromogenix) with a standard curve generated by diluting recombinant B-domain deleted hFVIII, Xyntha® (Pfizer), into cell growth medium.

EXAMPLE 2

[0366] The FIX construct regulatory element unit (SEQ ID NOs:22 and 23) is composed of a 321 bp intron of the apolipoprotein E (ApoE) gene and a 397 bp promoter of the human alpha-1 antitrypsin (hAAT) gene. Overall, this unit contains 16 CpGs.

Design of CpG reduced promoters

[0367] Either the cytosine or the guanine of CpG sites were changed depending on the consensus sequences. If no potential transcription factor binding site was found, the cytosine (C) of the CpG dinucleotide was replaced with thymine (T). By doing so, the pyrimidine-purine structure was maintained. In some cases, the C nucleotide or the entire CpG dinucleotide was deleted, and in some cases, the guanine (G) of the CpG dinucleotide was replaced with alanine (A) or C.

[0368] Using this strategy, 22 different sequences were generated based on the ApoE/hAAT regulatory element. The sequences are illustrated, with and without 5' and 3' flanking restriction enzyme sites, in SEQ ID NOs:24-67 below.

Cloning

[0369] The different promoters were synthesized and cloned upstream of a codon-optimized sequence (SEQ ID NO:94) encoding hFIX.

Mouse studies

[0370] The potency of the human alpha-1 antitrypsin (hAAT) gene promoter was assessed by hydrodynamic delivery of plasmid constructs in 8 weeks old male wild-type C57BL/6 mice (Jackson Laboratories). Non-fasted plasma samples were collected in heparin, 24 hours after plasmid administration, via submandibular blood collection. Plasma was placed on ice and stored at -80 °C until analyzed. All animal work was performed in accordance with institutional guidelines and approved protocols.

Potency Study

[0371] Plasma collected was used to evaluate hFIX transgene expression.

[0372] Activity levels of human FIX were measured by activated partial thromboplastin time (aPTT) assay. The aPTT assay was performed by mixing sample plasma in a 1:1:1 volume-ratio with human FIX-deficient plasma (George King Bio-Medical, Inc.) and aPTT reagent (Trinity Biotech), followed by a 180s incubation period at 37 °C. Coagulation was initiated by addition of 25 mM calcium chloride. Time to clot formation was measured using a STart 4 coagulation instrument (Diagnostica Stago). A standard curve was generated with pooled normal plasma (George King Bio-Medical, Inc.) starting at a 1:5 dilution in TBS pH 7.4 (48 µL + 192 µL) followed by serial 1:2 dilutions (120 µL +120 µL). The human standard curve was used to calculate the activity of each sample at week 17 after AAV vector administration; activity in two untreated mice was also measured. FIX activity in untreated mice was averaged and then subtracted from the treated samples to calculate the extra (*i.e.*, human) activity due to the exogenous FIX protein. The data are shown in Figure 1.

EXAMPLE 3

[0373] The FVIII construct regulatory element unit (SEQ ID NO:2 and SEQ ID NO:3) is composed of a 225 bp TTR promoter. Overall, this unit contains 4 CpGs.

Design of CpG reduced TTR promoters

[0374] Either the cytosine or the guanine of CpG sites were changed depending on the consensus sequences. If no potential binding site was found, the cytosine of the CpG dinucleotide was replaced with thymine. By doing so, the pyrimidine-purine structure was maintained. Using this strategy, 5 novel sequences were generated based on the TTRm (SEQ ID NO:3) regulatory element. The CpG reduced TTR sequences, with and without restriction enzyme sites, are illustrated in SEQ ID NOs:4-13.

[0375] Another set of four different short TTR hybrid promoters were designed. Five different liver-specific promoters were assessed *in silico* for the presence of putative transcription factor binding sites within 1000 nucleotides of the transcriptional start site (TSS) for their native genes. Subsequently, the TTR hybrid promoters were assembled by choosing specific regions from the original native promoters and assembling them in tandem. The TTR hybrid sequences, with and without restriction enzyme sites, are illustrated in SEQ ID NOs:14-21 below.

Cloning

[0376] The different promoters were synthetized and cloned upstream to a codon-optimized nucleotide sequence (SEQ ID NO:77) encodinghFVIII-BDD.

Mouse studies

[0377] The potency of the TTR promoters was initially assessed by hydrodynamic delivery in 8 weeks old male wild-type C57BL/6 mice (Jackson laboratories). Non-fasted plasma samples were collected in heparin 24 hours after plasmid administration via submandibular blood collection. Plasma was placed on ice and stored at -80 °C until analyzed. For AAV delivery studies, the first 0.5 mL of blood was discarded, and the remaining sample was collected in EDTA, and processed to plasma. All animal work was performed in accordance with institutional guidelines and approved protocols.

hFVIII antigen levels in murine plasma

[0378] Levels of hFVIII transgene product in murine plasma were quantified using a sandwich-style ELISA as follows: first, the wells of a microtiter plate were coated with an anti-hFVIII capture antibody (Green Mountain Antibodies, diluted to 2 µg/mL). The following day, the plate was washed four times and blocked (6% BSA, 0.2% Tween 20 in PBS) for 30 minutes at room temperature. Pooled murine plasma was spiked with a known concentration of recombinant B-domain deleted hFVIII (XYNTHA Solofuse®) and was serially diluted (1:2) to generate an 8-point standard curve ranging from 300 ng/mL to 2.34 ng/mL. The limit of quantitation of the assay is 4.8 ng/mL. Three levels of quality control samples were prepared and included on each plate to assess assay performance. After addition of the samples to the wells, the plate was incubated at 37 °C for 1 hour and then washed four times. A biotinylated anti-hFVIII detection antibody (Green Mountain Antibodies, diluted to 1 µg/mL) was added to the plate for 1 hour at room temperature to bind to the captured hFVIII protein. Following washing, a peroxidase-conjugated streptavidin reagent (Thermo Fisher Scientific) at a 1:5000 dilution was added to the plate for 30 minutes at room temperature to bind to the biotinylated anti-hFVIII antibody. After washing the plate to remove unbound conjugated antibody, the peroxidase activity was revealed following a 15-minute incubation at room temperature with 3,3',5,5'-tetramethylbenzidine substrate (TMB). The reaction was stopped with TMB Stop Solution and the plate was read by an absorbance plate reader for optical density (OD). The absorbance values obtained are proportional to the concentration of hFVIII present in the plasma sample. The data are shown in Figures 2-5.

RNA isolation and qPCR

[0379] Brain, testes, kidney, spleen, and liver mouse tissues were harvested, rinsed with DPBS, cut/mashed into multiple tiny pieces, and ~30 mg was used for RNA isolation, performed as described in the kit protocol (RNeasy plus universal mini kit, Qiagen). RNA concentrations were measured using a Nanodrop 2000 instrument, and samples were diluted to 150 ng/μL in nuclease-free. DNase treatment was done using Turbo DNA free kit (Invitrogen) as per the manufacturer's instructions. For cDNA reaction, 200 ng of RNA were used as directed in the High Capacity cDNA Reverse Transcription Kit (ABI). The cDNA samples were diluted 5-fold and 20 ng of cDNA were used in the PCR reaction. Quantitative real-time PCR was performed using forward primer: 5'- TGAGGAGGCTGAAGACTATGA-3' (SEQ ID NO:95), reverse primer: 5'- CCACAGACCTGATCTGAATGAA-3' (SEQ ID NO:96), and a probe: 5'-56-FAM-TGGATGTGG/ZEN/TGAGGTTTGATGATGACA-3IABkFQ-3' (SEQ ID NO:97). The murine actB (Integrated DNA Technologies) gene served as the housekeeping gene for normalization. The data are shown in Figure 6.

EXAMPLE 4

[0380] Expression of Factor VIII was increased by altering the elements within the expression cassette that contribute to transgene expression. An intron-free version of a B-domain deleted hFVIII expressing AAV vector (AAV-INTL) was made and compared with an intron-containing version (AAV-WINT). The AAV-INTL hFVIII expression cassette (SEQ ID NO:1) contains the same elements as the AAV-WINT hFVIII expression cassette, except that AAV-WINT hFVIII has a synthetic intron (SEQ ID NO:93) positioned between a TTRm promoter and a transgene encoding B-domain deleted human Factor VIII (Figure 7).

EXAMPLE 5: Potency in mice

[0381] To evaluate the potency of the intron-free cassette (TTRm hFVIII intronless; SEQ ID NO:1) versus the cassette with the intron (TTRm hFVIII) in mammals, male C57BL/6 mice (Jackson Laboratories) of approximately 8 weeks of age were injected intravenously in the lateral tail vein with AAV encapsidated cassettes at dosages of 6.4e9 or 1.6e10 vg/mouse. Plasma was collected at several time points, as indicated (Figures 8 and 9), and assessed for circulating hFVIII levels by hFVIII ELISA.

[0382] Determination of hFVIII levels showed that TTRm hFVIII intronless exhibited a significant increase in potency versus TTRm hFVIII (with the intron) (Figures 8 and 9), and this effect was seen at both doses and time points tested (study #1). These results were repeated in a subsequent study, study #2, with the 1.6e10 vg/mouse dose with 10 mice in each group (Figure 10). Study #2 confirmed that TTRm hFVIII intronless (AAV-INTL) was more potent than the vector containing the synthetic intron (AAV-WINT), and that these differences were sustained for at least 8 weeks.

EXAMPLE 6: Potency in NHPs, Study 1

[0383] AAV vector potencies of AAV-INTL and AAV-WINT were compared in NHPs (study #1). 12 male cynomolgus monkeys (*Macaca fascicularis*) between the ages of 24 and 50 months, with weights between 2-6 kg, and negative for AAV neutralizing antibodies, were divided into 4 randomized groups and injected intravenously with a single dose of either AAV-WINT or AAV-INTL based on the dosing groups shown in Table 1. Subsequently, plasma samples were obtained weekly to determine levels of circulating hFVIII.

Table 1. Group Designation and Dose Levels from NHP Study

Group #	No. of Animals (Male)	Dose Level (vg/kg)	Dose Concentration (vg/kg)
1 (AAV-WINT Low)	3	2.0×10^{12}	2.0×10^{11}
2 (AAV-WINT High)	3	6.0×10^{12}	6.0×10^{11}
3 (AAV-INTL Low)	3	2.0×10^{12}	2.0×10^{11}
4 (AAV-INTL High)	3	6.0×10^{12}	6.0×10^{11}

[0384] Levels of hFVIII in plasma of monkeys dosed with either AAV-WINT or AAV-INTL were determined by ELISA at weekly intervals throughout the 8-week study. At either dose tested in this study, 2e12 vg/kg (Figure 11) or 6e12 vg/kg (Figure 12), an increase in the levels of circulating hFVIII of from 2- to 4-fold based on peak circulating value, regardless of time point, was observed. As expected, we observed loss of expression 2-3 weeks following treatment, indicating the development of inhibitory antibodies to BDD hFVIII. The results of the first study demonstrate that AAV-INTL displays increased potency versus AAV-WINT in NHPs.

EXAMPLE 7: Potency in NHPs, Study 2

[0385] A second study (study #2) was undertaken in NHPs to confirm the increased vector potency of AAV-INTL versus AAV-WINT. Ten male cynomolgus macaques (*Macaca fascicularis*) between the ages of 24 and 50 months, with weights between 2-6 kg, and negative for AAV neutralizing antibodies, were divided into 2 randomized groups and injected intravenously with a single dose (2e12 vg/kg) of either AAV-WINT or AAV-INTL. Subsequently, plasma samples were obtained weekly to determine levels of circulating hFVIII.

[0386] Levels of hFVIII in plasma of macaques dosed with either AAV-WINT or AAV-INTLwere determined by ELISA at weekly intervals throughout the 8-week study. At the dose tested in this study, 2e12 vg/kg, an increase in the levels of circulating hFVIII of from 4- to 7-fold, based on peak circulating value, regardless of time point was observed (Figure 13). As previously seen in NHPs, loss of expression of the transgene 2-3 weeks following treatment was observed, due to the development of inhibitory antibodies to BDD hFVIII. The results of study #2 confirmed that AAV-INTL displays increased potency versus AAV-WINT in NHPs.

EXAMPLE 8: Determination of vector potency

[0387] To confirm that the correct vector, at the proper concentration, was dosed into each group of NHPs, dosing formulation titers were determined by qPCR, and the presence or absence of the synthetic intron in stock vectors was assayed by genotyping PCR assays that allow differentiation between AAV-WINT and AAV-INTL. To directly assess vector potency of both the undiluted stock vectors and the 2e12 vg/kg dosing formulations, a cell-based potency assay was utilized.

[0388] A human liver cell line was transduced with a dilution series of vector, and potency was determined by evaluating secreted BDD hFVIII in the supernatant by a hFVIII activity assay (Chromogenix Coatest SP4). At all MOI, AAV-INTL exhibited increased potency versus AAV-WINT (Figure 14). Notably, stock virus and dilution formulations showed similar potency within vector groups, further confirming that the titers of the dosing formulations in the 2e12 vg/kg group were properly prepared. In addition, at each MOI, when compared to AAV-WINT, AAV-INTL displayed an approximately 4-fold increase in potency

(Figure 15). These values are in agreement with the increased potency of AAV-INTLobserved in NHP studies 1 and 2.

EXAMPLE 9: Lot comparison

[0389] The first and second NHP studies used different lots of AAV-WINT and AAV-INTL vectors. To assess whether vector potency in the different lots was comparable, potency was measured using an *in vitro* assay (Figure 16). The results of these comparisons show that batch to batch variation was minimal and that AAV-INTL remained similarly about 4- to 5-fold more potent than AAV-WINT.

EXAMPLE 10: Determination of expression cassette efficiency

[0390] To explore the mechanism of increased potency *in vivo*, the transcriptional efficiency in the absence of viral transduction was determined. A human liver cell line was transfected with plasmids containing the expression cassettes that make up AAV-WINT and AAV-INTL, TTRm-intron-BDD-hFVIII and TTRm-BDD-hFVIII intronless (SEQ ID NO:1), respectively. Supernatants from these cells were assayed for hFVIII levels by a human FVIII activity assay (Chromogenix Coatest SP4).

[0391] Comparison of three independent DNA preparations of TTRm-intron-BDD-hFVIII and two independent DNA preparations of TTRm-BDD-hFVIII intronless (SEQ ID NO:1) displayed similar hFVIII levels, with a trend towards decreased expression upon intron removal (Figure 17). Although not wishing to be bound by any theory, the data suggest a non-transcriptional mechanism is driving the increased potency of AAV-INTL over AAV-WINT.

EXAMPLE 11: Discussion of Data

[0392] At equivalent doses, AAV-INTL exhibits increased potency and expression of BDD-hFVIII in cell culture, mouse and NHP models, over AAV-WINT. Mechanistically, increased potency was not apparently due to increased transcription of the FVIII transgene from the expression cassette, and is instead, perhaps, due to increased viral packaging efficiency or alternate mechanisms. These results indicate that intron-free expression cassettes may have increased potency in human clinical trials and provide benefits to patient safety and efficacy.

EXAMPLE 12: Human Clinical Trial Results

[0393] A single dose study was performed in four men (N = 4) with hemophila A, outlined in Table 4. All four participants received a single infusion of AAV-INTL hFVIII-BDD expression cassette (SEQ ID NO:1) encapsidated in LK03 AAV vector (SEQ ID NO:91), refered to herein as "LK03-INTL hFVIII-BDD," at a dose of $5x10^{11}$ vg/kg.

Table 4.

Participant		Age (yrs)	Weeks of Follow-up Post Infusion with Vector
1	840-09-601	28	21
2	840-02-601	18	17
3	840-02-602	63	9
4	840-10-601	63	5

[0394] LK03-INTL hFVIII-BDD vector was seen to drive FVIII expression in all four participants (Figs. 18-19).

[0395]

EXAMPLE 13: Sequences

Table 2. SEQ ID NOs and descriptions

SEQ ID NO	Description
SEQ ID NO:1	Entire nucleic acid sequence of AAV-INTL cassette (5' ITR, TTRm, hFVIII-BDD coding sequence (SEQ ID NO:77), PolyA, and 3'ITR).
SEQ ID NO:2	Nucleic acid sequence of the wild-type TTR promoter.
SEQ ID NO:3	Nucleic acid sequence of the mutated TTR promoter "TTRm", having 4 nucleotide changes from wild-type.
SEQ ID NO:4	Nucleic acid sequence of the **CpG1-TTRm** promoter, where every C is changed to T for all four CpGs.
SEQ ID NO:5	Nucleic acid sequence of the **CpG1-TTRm** promoter, where every C is changed to T for all four CpGs with restriction enzyme sites.

SEQ ID NO:6	Nucleic acid sequence of the **CpG2-TTRm** promoter, where the C is changed to T in the second, third and fourth CpG.
SEQ ID NO:7	Nucleic acid sequence of the **CpG2-TTRm** promoter, where the C is changed to T in the second, third and fourth CpG with restriction enzyme sites.
SEQ ID NO:8	Nucleic acid sequence of the **CpG3-TTRm** promoter, where the C is changed to T in the first, third and fourth CpG.
SEQ ID NO:9	Nucleic acid sequence of the **CpG3-TTRm** promoter, where the C is changed to T in the first, third and fourth CpG with restriction enzyme sites.
SEQ ID NO:10	Nucleic acid sequence of the **CpG4-TTRm** promoter, where the C is changed to T in the first, second and fourth CpG.
SEQ ID NO:11	Nucleic acid sequence of the **CpG4-TTRm** promoter, where the C is changed to T in the first, second and fourth CpG with restriction enzyme sites.
SEQ ID NO:12	Nucleic acid sequence of the **CpG5-TTRm** promoter, where the C is changed to T in the first, second and third CpG.
SEQ ID NO:13	Nucleic acid sequence of the **CpG5-TTRm** promoter, where the C is changed to T in the first, second and third CpG with restriction enzyme sites.
SEQ ID NO:14	Nucleic acid sequence of the **Hybrid6** promoter.
SEQ ID NO:15	Nucleic acid sequence of the **Hybrid6** promoter with restriction enzyme sites.
SEQ ID NO:16	Nucleic acid sequence of the **Hybrid7** promoter.
SEQ ID NO:17	Nucleic acid sequence of the **Hybrid7** promoter with restriction enzyme sites.
SEQ ID NO:18	Nucleic acid sequence of the **Hybrid8** promoter.
SEQ ID NO:19	Nucleic acid sequence of the **Hybrid8** promoter with restriction enzyme sites.
SEQ ID NO:20	Nucleic acid sequence of the **Hybrid9** promoter.
SEQ ID NO:21	Nucleic acid sequence of the **Hybrid9** promoter with restriction enzyme sites.
SEQ ID NO:22	Nucleic acid sequence of the non-CpG reduced ApoE/hAAT regulatory element.
SEQ ID NO:23	Nucleic acid sequence of the non-CpG reduced ApoE/hAAT regulatory element with flanking restriction enzyme sites.
SEQ ID NO:24	Nucleic acid sequence of the **CpG1- ApoE/hAAT** promoter**.**
SEQ ID NO:25	Nucleic acid sequence of the **CpG1- ApoE/hAAT** promoter with restriction enzyme sites.

SEQ ID NO:26	Nucleic acid sequence of the **CpG2- ApoE/hAAT** promoter.
SEQ ID NO:27	Nucleic acid sequence of the **CpG2- ApoE/hAAT** promoter with restriction enzyme sites.
SEQ ID NO:28	Nucleic acid sequence of the **CpG3- ApoE/hAAT** promoter.
SEQ ID NO:29	Nucleic acid sequence of the **CpG3- ApoE/hAAT** promoter with restriction enzyme sites.
SEQ ID NO:30	Nucleic acid sequence of the **CpG4- ApoE/hAAT** promoter.
SEQ ID NO:31	Nucleic acid sequence of the **CpG4- ApoE/hAAT** promoter with restriction enzyme sites.
SEQ ID NO:32	Nucleic acid sequence of the **CpG5- ApoE/hAAT** promoter.
SEQ ID NO:33	Nucleic acid sequence of the **CpG5- ApoE/hAAT** promoter with restriction enzyme sites.
SEQ ID NO:34	Nucleic acid sequence of the **CpG6- ApoE/hAAT** promoter.
SEQ ID NO:35	Nucleic acid sequence of the **CpG6- ApoE/hAAT** with restriction enzyme sites.
SEQ ID NO:36	Nucleic acid sequence of the **CpG7- ApoE/hAAT** promoter.
SEQ ID NO:37	Nucleic acid sequence of the **CpG7- ApoE/hAAT** promoter with restriction enzyme sites.
SEQ ID NO:38	Nucleic acid sequence of the **CpG8- ApoE/hAAT** promoter.
SEQ ID NO:39	Nucleic acid sequence of the **CpG8- ApoE/hAAT** promoter with restriction enzyme sites.
SEQ ID NO:40	Nucleic acid sequence of the **CpG9- ApoE/hAAT** promoter.
SEQ ID NO:41	Nucleic acid sequence of the **CpG9- ApoE/hAAT** promoter with restriction enzyme sites
SEQ ID NO:42	Nucleic acid sequence of the **CpG10- ApoE/hAAT** promoter.
SEQ ID NO:43	Nucleic acid sequence of the **CpG10- ApoE/hAAT** promoter with restriction enzyme sites
SEQ ID NO:44	Nucleic acid sequence of the **CpG11- ApoE/hAAT** promoter.
SEQ ID NO:45	Nucleic acid sequence of the **CpG11- ApoE/hAAT** promoter with restriction enzyme sites.
SEQ ID NO:46	Nucleic acid sequence of the **CpG12- ApoE/hAAT** promoter.
SEQ ID NO:47	Nucleic acid sequence of the **CpG12- ApoE/hAAT** promoter with restriction enzyme sites.
SEQ ID NO:48	Nucleic acid sequence of the **CpG13- ApoE/hAAT** promoter.
SEQ ID NO:49	Nucleic acid sequence of the **CpG13- ApoE/hAAT** promoter with restriction enzyme sites.
SEQ ID NO:50	Nucleic acid sequence of the **CpG14- ApoE/hAAT** promoter.

SEQ ID NO:51	Nucleic acid sequence of the **CpG14- ApoE/hAAT** promoter with restriction enzyme sites.
SEQ ID NO:52	Nucleic acid sequence of the **CpG15- ApoE/hAAT** promoter.
SEQ ID NO:53	Nucleic acid sequence of the **CpG15- ApoE/hAAT** promoter with restriction enzyme sites.
SEQ ID NO:54	Nucleic acid sequence of the **CpG16- ApoE/hAAT** promoter.
SEQ ID NO:55	Nucleic acid sequence of the **CpG16- ApoE/hAAT** promoter with restriction enzyme sites.
SEQ ID NO:56	Nucleic acid sequence of the **CpG17- ApoE/hAAT** promoter.
SEQ ID NO:57	Nucleic acid sequence of the **CpG17- ApoE/hAAT** promoter with restriction enzyme sites.
SEQ ID NO:58	Nucleic acid sequence of the **CpG18- ApoE/hAAT** promoter.
SEQ ID NO:59	Nucleic acid sequence of the **CpG18- ApoE/hAAT** promoter with restriction enzyme sites.
SEQ ID NO:60	Nucleic acid sequence of the **CpG19- ApoE/hAAT** promoter.
SEQ ID NO:61	Nucleic acid sequence of the **CpG19- ApoE/hAAT** promoter with restriction enzyme sites.
SEQ ID NO:62	Nucleic acid sequence of the **CpG20- ApoE/hAAT** promoter.
SEQ ID NO:63	Nucleic acid sequence of the **CpG20- ApoE/hAAT** promoter with restriction enzyme sites.
SEQ ID NO:64	Nucleic acid sequence of the **CpG21- ApoE/hAAT** promoter.
SEQ ID NO:65	Nucleic acid sequence of the **CpG21- ApoE/hAAT** promoter with restriction enzyme sites.
SEQ ID NO:66	Nucleic acid sequence of the **CpG22- ApoE/hAAT** promoter.
SEQ ID NO:67	Nucleic acid sequence of the **CpG22- ApoE/hAAT** promoter with restriction enzyme sites.
SEQ ID NO:68	The amino acid sequence of FVIII-BDD.
SEQ ID NO:69	The amino acid sequence of SFSQNPPVLKRHQR (“SQ sequence”).
SEQ ID NO:70	The wild-type nucleic acid sequence encoding FVIII-BDD.
SEQ ID NO:71	Nucleic acid sequence encoding FVIII-BDD.
SEQ ID NO:72	Nucleic acid sequence encoding FVIII-BDD.
SEQ ID NO:73	Nucleic acid sequence encoding FVIII-BDD.
SEQ ID NO:74	Nucleic acid sequence encoding FVIII-BDD.
SEQ ID NO:75	Nucleic acid sequence encoding FVIII-BDD.
SEQ ID NO:76	Nucleic acid sequence encoding FVIII-BDD.
SEQ ID NO:77	Nucleic acid sequence encoding FVIII-BDD.

SEQ ID NO:78	Nucleic acid sequence encoding FVIII-BDD.
SEQ ID NO:79	Nucleic acid sequence encoding FVIII-BDD.
SEQ ID NO:80	Nucleic acid sequence encoding FVIII-BDD.
SEQ ID NO:81	Nucleic acid sequence encoding FVIII-BDD.
SEQ ID NO:82	Nucleic acid sequence encoding FVIII-BDD.
SEQ ID NO:83	Nucleic acid sequence encoding FVIII-BDD.
SEQ ID NO:84	Nucleic acid sequence encoding FVIII-BDD.
SEQ ID NO:85	Nucleic acid sequence encoding FVIII-BDD.
SEQ ID NO:86	Nucleic acid sequence encoding FVIII-BDD.
SEQ ID NO:87	Nucleic acid sequence encoding FVIII-BDD.
SEQ ID NO:88	Nucleic acid sequence encoding FVIII-BDD.
SEQ ID NO:89	cDNA encoding FVIII-V3
SEQ ID NO:90	cDNA encoding FVIII-CO3
SEQ ID NO:91	LK03 capsid protein
SEQ ID NO:92	SPK capsid protein
SEQ ID NO:93	Nucleic acid sequence of intron in AAV-WINT cassette.
SEQ ID NO:94	Nucleic acid encoding human FIX
SEQ ID NO:95	Forward primer
SEQ ID NO:96	Reverse primer
SEQ ID NO:97	Probe

Entire nucleic acid sequence of AAV-INTL expression cassette (5' ITR, TTRm, hFVIII-BDD, PolyA, and 3'ITR) (SEQ ID NO:1) and legend (Table 3):

cctgcaggcagctgcgcgctcgctcgctcactgaggccgcccgggcaaagcccgggcgtcgg
gcgacctttggtcgcccggcctcagtgagcgagcgagcgcgcagagagggagtggccaactc
catcactaggggttcctgtcgacgtgtctgtctgcacatttcgtagagcgagtgttccgata
ctctaatctccctaggcaaggttcatattgacttaggttacttattctccttttgttgacta
agtcaataatcagaatcagcaggtttggagtcagcttggcagggatcagcagcctgggttgg
aaggaggggtataaaagccccttcaccaggagaagccgtcacacagatccacaagctcctg
ctagcgtttaaacgccaccatgcagattgagctgagcacctgcttcttcctgtgtctgctga
ggttctgcttctctgccaccaggaggtattacctgggggctgtggagctgagctgggactat
atgcagtctgacctgggggagctgcctgtggatgctaggttcccccccagggtgcccaagag
cttccccttaacacttctgtggtgtacaagaagaccctgtttgtggagttcactgaccacc
tgttcaacattgccaagcccaggcccccctggatggggctgctggggcccaccatccaggct
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tgctgtgggggtgagctactggaaggcttctgaggggctgagtatgatgaccagactagcc
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tgtggacctggtgaaggacctgaactctggcctgattggggctctgctggtgtgtagggagg
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ctctgccagggcttggcctaagatgcacactgtgaatgggtatgtgaataggagcctgcctg
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tcattcagatcaggtctgtggccaagaaacaccccaagacctgggtgcactacattgctgct
gaggaagaggactgggactatgctcccctggtgctggcccctgatgataggtcttataagag
ccagtacctgaacaatgggccccagaggattggcaggaagtacaagaaggtgaggttcatgg
cctacactgatgaaaccttcaaaaccagggaggccattcagcatgagtctggcatcctgggc
cctctgctgtatggggaggtgggggacaccctgctgatcatcttcaagaaccaggccagcag
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gcatcaatggctatgtgtttgacagcctgcagctgtctgtgtgcctgcatgaggtggcctac
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cttcaagcacaagatggtgtatgaggataccctgaccctgttccccttctctggggagactg
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aacaggggatgactgccctgctgaaggtgtctagctgtgataagaacactggggactacta
tgaggacagctatgaggacatttctgcttatctgctgtctaagaataatgccattgagccca
gaagcttcagccagaatccccctgtgctgaagagacatcagagggagatcaccagaactacc
ctgcagtctgatcaggaggagattgactatgatgacactatctctgtggagatgaagaagga
ggactttgacatctatgatgaggatgagaatcagtctcccaggagctttcagaagaagacca
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tgctgagcatgggctctaatgagaatatccacagcatccacttctctgggcatgtgttcact
gtgaggaagaaggaggagtacaagatggctctgtataatctgtaccctggggtgtttgaaac
tgtggagatgctgcccctctaaggctggcatctggagggtggagtgcctgattggggagcacc

tgcatgctggcatgagcaccctgttcctggtgtacagcaacaagtgccagacccccctgggc
atggcctctggccacatcagggacttccagatcactgcctctggccagtatggccagtgggc
ccccaagctggccaggctgcactattctggcagcatcaatgcctggagcaccaaggagccct
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caggggaggagcaatgcctggaggcctcaggtgaacaaccccaaggagtggctgcaggtgga
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cagcctggacccccccctgctgaccagatacctgaggatccacccccagtcttgggtgcatc
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ataaaagatcagagctctagagatctgtgtgttggtttttgtgtaggaacccctagtgatg
gagttggccactccctctctgcgcgctcgctcgctcactgaggccgggcgaccaaaggtcgc
ccgacgcccgggctttgcccgggcggcctcagtgagcgagcgagcgcgcagctgcctgcagg

Table 3. Features of SEQ ID NO:1

Name	Type	Start	End
AAV2 5' ITR	repeat region	1	141
TTRm promoter	promoter	148	372
B-domain deleted, codon-optimized hFVIII	coding sequence	392	4,765
Rabbit beta-globin polyA	poly A signal	4,774	4,819
AAV2 3' ITR	repeat region	4,820	4,960

Wild-type TTR promoter (SEQ ID NO:2). The 4 underlined nucleotides are altered in mutated TTR promoter (SEQ ID NO:3), below.

gtgtctgtctgcacatttcgtagagcgagtgttccgatactctaatctccctaggcaaggtt
catatttgtgtaggttacttattctccttttgttgactaagtcaataatcagaatcagcagg
tttggagtcagcttggcagggatcagcagcctgggttggaaggaggggtataaaagcccct
tcaccaggagaagccgtcacacagatccacaagctcctg

Mutated TTR promoter (4 nucleotide changes; underlined) "TTRm" (SEQ ID NO:3):

gtgtctgtctgcacatttcgtagagcgagtgttccgatactctaatctccctaggcaaggtt
catattgacttaggttacttattctccttttgttgactaagtcaataatcagaatcagcagg
tttggagtcagcttggcagggatcagcagcctgggttggaaggaggggtataaaagcccct
tcaccaggagaagccgtcacacagatccacaagctcctg

Nucleic acid sequence of CpG1-TTRm (SEQ ID NO:4). In CpG1, every C is changed to T for all four CpGs (double underlined). MluI (acgcgt) and PmeI (gtttaaac) restriction sites are at the 5' and 3' ends, respectively, when the sequence was cloned into the FVIII expression cassette. SEQ ID NO:5 is CpG1-TTRm with these restriction enzyme sites (underlined).

SEQ ID NO:4:

gtgtctgtctgcacattttgtagagtgagtgttctgatactctaatctccctagg
caaggttcatattgacttaggttacttattctcctttgttgactaagtcaataa
tcagaatcagcaggtttggagtcagcttggcagggatcagcagcctgggttggaa
ggaggggtataaaagcccttcaccaggagaagctgtcacacagatccacaagc
tcctg

SEQ ID NO:5:

acgcgtgtctgtctgcacattttgtagagtgagtgttctgatactctaatctccc
taggcaaggttcatattgacttaggttacttattctcctttgttgactaagtca
ataatcagaatcagcaggtttggagtcagcttggcagggatcagcagcctgggtt
ggaaggaggggtataaaagcccttcaccaggagaagctgtcacacagatccac
aagctcctgtttaaac

Nucleic acid sequence of CpG2-TTRm (SEQ ID NO:6). In CpG2, the C is changed to T in the second, third and fourth CpG (double underlined). MluI (acgcgt) and PmeI (gtttaaac) restriction sites are at the 5' and 3' ends, respectively, when the sequence was cloned into the FVIII expression cassette. SEQ ID NO:7 is CpG2-TTRm with these restriction enzyme sites (underlined).

SEQ ID NO:6:

gtgtctgtctgcacatttcgtagagtgagtgttctgatactctaatctccctagg
caaggttcatattgacttaggttacttattctcctttgttgactaagtcaataa
tcagaatcagcaggtttggagtcagcttggcagggatcagcagcctgggttggaa
ggaggggtataaaagcccttcaccaggagaagctgtcacacagatccacaagc
tcctg

SEQ ID NO:7:

acgcgtgtctgtctgcacatttcgtagagtgagtgttctgatactctaatctccc
taggcaaggttcatattgacttaggttacttattctcctttgttgactaagtca
ataatcagaatcagcaggtttggagtcagcttggcagggatcagcagcctgggtt
ggaaggaggggtataaaagcccttcaccaggagaagctgtcacacagatccac
aagctcctgtttaaac

Nucleic acid sequence of CpG3-TTRm (SEQ ID NO:8). In CpG3, the C is changed to T in the first, third and fourth CpG (double underlined). MluI (acgcgt) and PmeI (gtttaaac) restriction sites are at the 5' and 3' ends, respectively, when the sequence was cloned into the

FVIII expression cassette. SEQ ID NO:9 is CpG3-TTRm with these restriction enzyme sites (underlined).

SEQ ID NO:8:

gtgtctgtctgcacatttgtagagcgagtgttctgatactctaatctccctagg
caaggttcatattgacttaggttacttattctcctttgttgactaagtcaataa
tcagaatcagcaggtttggagtcagcttggcagggatcagcagcctgggttggaa
ggaggggtataaaagccccttcaccaggagaagctgtcacacagatccacaagc
tcctg

SEQ ID NO:9:

acgcgtgtctgtctgcacatttgtagagcgagtgttctgatactctaatctccc
taggcaaggttcatattgacttaggttacttattctcctttgttgactaagtca
ataatcagaatcagcaggtttggagtcagcttggcagggatcagcagcctgggtt
ggaaggaggggtataaaagccccttcaccaggagaagctgtcacacagatccac
aagctcctgtttaaac

Nucleic acid sequence of CpG4-TTRm (SEQ ID NO:10). In CpG4, the C is changed to T in the first, second and fourth CpG (double underlined). MluI (acgcgt) and PmeI (gtttaaac) restriction sites are at the 5' and 3' ends, respectively, when the sequence was cloned into the FVIII expression cassette. SEQ ID NO:11 is CpG4-TTRm with these restriction enzyme sites (underlined).

SEQ ID NO:10:

gtgtctgtctgcacatttgtagagtgagtgttccgatactctaatctccctagg
caaggttcatattgacttaggttacttattctcctttgttgactaagtcaataa
tcagaatcagcaggtttggagtcagcttggcagggatcagcagcctgggttggaa
ggaggggtataaaagccccttcaccaggagaagctgtcacacagatccacaagc
tcctg

SEQ ID NO:11:

acgcgtgtctgtctgcacatttgtagagtgagtgttccgatactctaatctccc
taggcaaggttcatattgacttaggttacttattctcctttgttgactaagtca
ataatcagaatcagcaggtttggagtcagcttggcagggatcagcagcctgggtt
ggaaggaggggtataaaagccccttcaccaggagaagctgtcacacagatccac
aagctcctgtttaaac

Nucleic acid sequence of CpG5-TTRm (SEQ ID NO:12). In CpG5, the C is changed to T in the first, second and third CpG (double underlined). MluI (acgcgt) and PmeI (gtttaaac) restriction sites are at the 5' and 3' ends, respectively, when the sequence was cloned into the FVIII expression cassette. SEQ ID NO:13 is CpG5-TTRm with these restriction enzyme sites (underlined).

SEQ ID NO:12:

gtgtctgtctgcacatttgtagagtgagtgttctgatactctaatctccctagg
caaggttcatattgacttaggttacttattctcctttgttgactaagtcaataa
tcagaatcagcaggtttggagtcagcttggcagggatcagcagcctgggttggaa

ggaggggtataaaagcccttcaccaggagaagccgtcacacagatccacaagc
tcctg

SEQ ID NO:13:

acgcgtgtctgtctgcacatttgtagagtgagtgttctgatactctaatctccc
taggcaaggttcatattgacttaggttacttattcctttttgttgactaagtca
ataatcagaatcagcaggtttggagtcagcttggcagggatcagcagcctgggtt
ggaaggaggggtataaaagcccttcaccaggagaagccgtcacacagatccac
aagctcctgtttaaac

Nucleic acid sequence of Hybrid6 promoter (TTR/hAAT/albumin hybrid) (SEQ ID NO:14). The G is changed to A in the single CpG dinucleotide (double underlined). MluI (acgcg) and PmeI (gtttaaac) restriction sites are at the 5' and 3' ends, respectively, when the sequence was cloned into the FVIII expression cassette. (Italics=TTR, Underline=albumin, Bold=hAAT.) SEQ ID NO:15 is Hybrid6 promoter with these restriction enzyme sites.

*TAGGCAAGGTTCATATTGACTTAGGTTACTTATTCTCCTTT*TGCCTGCTGACCTTGGAGCTG

GGGCAGAGGTCAGAG*GAGTCAGCTTGGCAGGGATCAGCAG***ATGAATTTTGTAATCAGTTCCC**

TTGAGTCATTAAAAAATATAAAA**CAAAGATGAGTCTAGTTAATAATCTACAAT**

Nucleic acid sequence of Hybrid7 promoter (TTR/hAAT hybrid) (SEQ ID NO:16). The C is changed to T in both CpG dinucleotides (double undelined). MluI (acgcg) and PmeI (gtttaaac) restriction sites are at the 5' and 3' ends, respectively, when the sequence was cloned into the FVIII expression cassette. (Italics=TTR, Bold=hAAT.) SEQ ID NO:17 is Hybrid7 promoter with these restriction enzyme sites.

*TAGGCAAGGTTCATATTGACTTAGGTTACTTATTCTCCTTT***TGATAACTGGGGTG**

ACCTTGGTTAATATTCACCAGCA*GAGTCAGCTTGGCAGGGATCAGCAGCCTGGGT*

*TGGAAGGAGGGGGTATAAAA***TGATAACTGGGGTGACCTTGGTTAATATTCACCAG**

CA

Nucleic acid sequence of Hybrid8 promoter (TTR/FGG (fribrinogen gamma chain gene promoter)/albumin promoter hybrid) (SEQ ID NO:18). The G is changed to A in the single CpG dinucleotide (double underlined). MluI (acgcgt) and PmeI (gtttaaac) restriction sites are at the 5' and 3' ends, respectively, when the sequence was cloned into the FVIII expression cassette. (Underline=albumin, Underline italics=FGG, Italics=TTR.) SEQ ID NO:19 is Hybrid8 promoter with these restriction enzyme sites.

ACTCCCTTGAGTCATTAAAAAAATATATTTGGTAATTCATAAACCTTACAAACATTTACTTA

*ACACTTACCATGAATTGGGTAATGTGCTCA**ATTGACTTAGGTTACTTATTCTCCTTT***TGAAT

TTTTTGGCAAGAATATTATGAATTTTGTAATCAGTTATAAAGGCAGCCAATGAAATACAAAG

ATGAGTCTAGTTAATAATCTACAAT

Nucleic acid sequence of Hybrid9 promoter (TTR/FGG/hAAT/SAA1 hybrid) (SEQ ID NO:20). The C is changed to T in all three CpG dinucleotides (double underlined). MluI (acgcgt) and PmeI (tttaaac) restriction sites at the 5' and 3' ends, respectively, when the sequence was cloned into the FVIII expression cassette. (Italics= TTR, Bold=hAAT, Underline italics=FGG, Underline bold=SAA1.) SEQ ID NO:21 is Hybrid9 promoter with these restriction enzyme sites.

*TAGGCAAGGTTCATATTGACTTAGGTTACTTATTCTCCTTT***GGGTGACTCAGATCCCAGCCA GTGGACTTAGCCCCTGTTTGCTCCTCTGATAACTGGGGTGACCTTGGTTAATATTCACCAGC A***TTTTTGAGTCAATAATAATGTTAACTGATCCCTAGGCTA**TA*AAATAATAGTGTTAACTGAT CCCT***GTGCAGTGGTGTGATTATAG**

Nucleic acid sequence of non-CpG reduced ApoE/hAAT regulatory element (SEQ ID NO:22). The sequence contains a total of CpGs (double underlined). The C/EBP (CCAAT/enhancer-binding protein) site is underlined.

atgccacctccaacatccactcgacccccttggaatttcggtggagaggagcagag
gttgtcctggcgtggtttaggtagtgtgagaggggtaccggggatcttgctacc
agtggaacagccactaaggattctgcagtgagagcagagggccagctaagtggta
ctctcccagagactgtctgactcacgccacccccctccaccttggacacaggacgc
tgtggtttctgagccaggtacaatgactcctttcggtaagtgcagtggaagctgt
acactgcccaggcaaagcgtccgggcagcgtaggcgggcgactcagatcccagcc
agtggacttagcccctgtttgctcctccgataactggggtgaccttggttaatat
tcaccagcagcctccccgttgcccctctggatccactgcttaaatacggacgag
gaca

Nucleic acid sequence of non-CpG reduced ApoE/hAAT regulatory unit flanked at the 5' and 3' ends by ApaI restriction sites (SEQ ID NO:23). The sequence contains a total of 16 CpGs (double underlined). The ApaI restriction sites were used when the sequence was cloned into the FIX expression cassette.

gggcccatgccacctccaacatccactcgacccccttggaatttcggtggagagga
gcagaggttgtcctggcgtggtttaggtagtgtgagaggggtaccggggatctt
gctaccagtggaacagccactaaggattctgcagtgagagcagagggccagctaa
gtggtactctcccagagactgtctgactcacgccacccccctccaccttggacaca
ggacgctgtggtttctgagccaggtacaatgactcctttcggtaagtgcagtgga
agctgtacactgcccaggcaaagcgtccgggcagcgtaggcgggcgactcagatc
ccagccagtggacttagcccctgtttgctcctccgataactggggtgaccttggt
taatattcaccagcagcctccccgttgcccctctggatccactgcttaaatacg
gacgaggacagggccc

Nucleic acid sequence of CpG1-ApoE/hAAT (SEQ ID NO:24). Every C changed to T, except in the seventh CpG, where G is changed to A. ApaI restriction sites are at the 5' and 3' ends when the sequence was cloned into the FIX expression cassette. SEQ ID NO:25 is CpG1-ApoE/hAAT with these restriction enzyme sites (underlined).

gggcccatgccacctccaacatccactgacccttggaatttggtggagagga
gcagaggttgtcctggtgtggtttaggtagtgtgagaggggtacctggggatctt
gctaccagtggaacagccactaaggattctgcagtgagagcagagggccagctaa
gtggtactctcccagagactgtctgactcatgccacccccctccaccttggacaca
ggatgctgtggtttctgagccaggtacaatgactcctttcagtaagtgcagtgga
agctgtacactgcccaggcaaagtgtctgggcagtgtaggtgggtgactcagatc
ccagccagtggacttagcccctgtttgctcctctgataactggggtgaccttggt
taatattcaccagcagcctccccctgttgcccctctggatccactgcttaaatatg
gatgaggacagggccc

Nucleic acid sequence of CpG2-ApoE/hAAT (SEQ ID NO:26). Every C changed to T, except leave first CpG with no change, and G changed to A in the seventh CpG. The unchanged "c" is in bold. ApaI restriction sites are at the 5' and 3' ends, respectively, when the sequence was cloned into the FIX expression cassette. SEQ ID NO:27 is CpG2-ApoE/hAAT with these restriction enzyme sites (underlined).

gggcccatgccacctccaacatccact**c**gacccttggaatttggtggagagga
gcagaggttgtcctggtgtggtttaggtagtgtgagaggggtacctggggatctt
gctaccagtggaacagccactaaggattctgcagtgagagcagagggccagctaa
gtggtactctcccagagactgtctgactcatgccacccccctccaccttggacaca
ggatgctgtggtttctgagccaggtacaatgactcctttcagtaagtgcagtgga
agctgtacactgcccaggcaaagtgtctgggcagtgtaggtgggtgactcagatc
ccagccagtggacttagcccctgtttgctcctctgataactggggtgaccttggt
taatattcaccagcagcctccccctgttgcccctctggatccactgcttaaatatg
gatgaggacagggccc

Nucleic acid sequence of CpG3-ApoE/hAAT (SEQ ID NO:28). Every C changed to T, except leave second CpG with no change, and G changed to A in the seventh CpG. The unchanged "c" is in bold. ApaI restriction sites are at the 5' and 3' ends when the sequence was cloned into the FIX expression cassette. SEQ ID NO:29 is CpG3-ApoE/hAAT with these restriction enzyme sites (underlined).

gggcccatgccacctccaacatccactgacccttggaattt**c**ggtggagagga
gcagaggttgtcctggtgtggtttaggtagtgtgagaggggtacctggggatctt
gctaccagtggaacagccactaaggattctgcagtgagagcagagggccagctaa
gtggtactctcccagagactgtctgactcatgccacccccctccaccttggacaca
ggatgctgtggtttctgagccaggtacaatgactcctttcagtaagtgcagtgga
agctgtacactgcccaggcaaagtgtctgggcagtgtaggtgggtgactcagatc
ccagccagtggacttagcccctgtttgctcctctgataactggggtgaccttggt
taatattcaccagcagcctccccctgttgcccctctggatccactgcttaaatatg
gatgaggacagggccc

Nucleic acid sequence of CpG4-ApoE/hAAT (SEQ ID NO:30). Every C changed to T, except leave third CpG with no change, and G changed to A in the seventh CpG. The unchanged "c" is in bold. ApaI restriction sites are at the 5' and 3' ends when the sequence was cloned into the FIX expression cassette. SEQ ID NO:31 is CpG4-ApoE/hAAT with these restriction enzyme sites (underlined).

gggcccatgccacctccaacatccacttgacccctttggaatttttggtggagagga
gcagaggttgtcctggcgtggtttaggtagtgtgagaggggtacctggggatctt
gctaccagtggaacagccactaaggattctgcagtgagagcagagggccagctaa
gtggtactctcccagagactgtctgactcatgccacccccctccaccttggacaca
ggatgctgtggtttctgagccaggtacaatgactcctttcagtaagtgcagtgga
agctgtacactgcccaggcaaagtgtctgggcagtgtaggtgggtgactcagatc
ccagccagtggacttagcccctgtttgctcctctgataactggggtgaccttggt
taatattcaccagcagcctcccctgttgcccctctggatccactgcttaaatatg
gatgaggacagggccc

Nucleic acid sequence of CpG5-ApoE/hAAT (SEQ ID NO:32). Every C changed to T, except leave fourth CpG with no change, and G changed to A in the seventh CpG. The unchanged CpG (the fourth) is in bold. ApaI restriction sites are at the 5' and 3' ends when the sequence was cloned into the FIX expression cassette. SEQ ID NO:33 is CpG5-ApoE/hAAT with these restriction enzyme sites (underlined).

gggcccatgccacctccaacatccacttgacccctttggaatttttggtggagagga
gcagaggttgtcctggtgtggtttaggtagtgtgagaggggtaccgggatctt
gctaccagtggaacagccactaaggattctgcagtgagagcagagggccagctaa
gtggtactctcccagagactgtctgactcatgccacccccctccaccttggacaca
ggatgctgtggtttctgagccaggtacaatgactcctttcagtaagtgcagtgga
agctgtacactgcccaggcaaagtgtctgggcagtgtaggtgggtgactcagatc
ccagccagtggacttagcccctgtttgctcctctgataactggggtgaccttggt
taatattcaccagcagcctcccctgttgcccctctggatccactgcttaaatatg
gatgaggacagggccc

Nucleic acid sequence of CpG6-ApoE/hAAT (SEQ ID NO:34). Every C changed to T, except leave fifth CpG with no change, and G changed to A in the seventh CpG. The unchanged CpG (the fifth) is in bold. ApaI restriction sites are at the 5' and 3' ends when the sequence was cloned into the FIX expression cassette. SEQ ID NO:35 is CpG6-ApoE/hAAT with these restriction enzyme sites (underlined).

gggcccatgccacctccaacatccacttgacccctttggaatttttggtggagagga
gcagaggttgtcctggtgtggtttaggtagtgtgagaggggtacctggggatctt
gctaccagtggaacagccactaaggattctgcagtgagagcagagggccagctaa
gtggtactctcccagagactgtctgactcacgccacccccctccaccttggacaca
ggatgctgtggtttctgagccaggtacaatgactcctttcagtaagtgcagtgga
agctgtacactgcccaggcaaagtgtctgggcagtgtaggtgggtgactcagatc
ccagccagtggacttagcccctgtttgctcctctgataactggggtgaccttggt
taatattcaccagcagcctcccctgttgcccctctggatccactgcttaaatatg
gatgaggacagggccc

Nucleic acid sequence of CpG7-ApoE/hAAT (SEQ ID NO:36). Every C changed to T, except leave sixth CpG with no change, and G changed to A in the seventh CpG. The unchanged CpG (the sixth) is in bold. ApaI restriction sites are at the 5' and 3' ends when the sequence was cloned into the FIX expression cassette. SEQ ID NO:37 is CpG7-ApoE/hAAT with these restriction enzyme sites (underlined).

gggcccatgccacctccaacatccactgacccttggaatttggtggagagga
gcagaggttgtcctggtgtggtttaggtagtgtgagaggggtacctggggatctt
gctaccagtggaacagccactaaggattctgcagtgagagcagagggccagctaa
gtggtactctcccagagactgtctgactcatgccacccccctccaccttggacaca
ggacgctgtggtttctgagccaggtacaatgactcctttcagtaagtgcagtgga
agctgtacactgcccaggcaaagtgtctgggcagtgtaggtgggtgactcagatc
ccagccagtggacttagcccctgtttgctcctctgataactggggtgaccttggt
taatattcaccagcagcctccccctgttgcccctctggatccactgcttaaatatg
gatgaggacagggccc

Nucleic acid sequence of CpG8-ApoE/hAAT (SEQ ID NO:38). Every C changed to T, except leave seventh site with no change. The unchanged CpG (the seventh) is in bold. ApaI restriction sites are at the 5' and 3' ends when the sequence was cloned into the FIX expression cassette. SEQ ID NO:39 is CpG8-ApoE/hAAT with these restriction enzyme sites (underlined).

gggcccatgccacctccaacatccactgacccttggaatttggtggagagga
gcagaggttgtcctggtgtggtttaggtagtgtgagaggggtacctggggatctt
gctaccagtggaacagccactaaggattctgcagtgagagcagagggccagctaa
gtggtactctcccagagactgtctgactcatgccacccccctccaccttggacaca
ggatgctgtggtttctgagccaggtacaatgactcctttcggtaagtgcagtgga
agctgtacactgcccaggcaaagtgtctgggcagtgtaggtgggtgactcagatc
ccagccagtggacttagcccctgtttgctcctctgataactggggtgaccttggt
taatattcaccagcagcctccccctgttgcccctctggatccactgcttaaatatg
gatgaggacagggccc

Nucleic acid sequence of CpG9-ApoE/hAAT (SEQ ID NO:40). Every C changed to T, except leave eighth CpG with no change, and G changed to A in the seventh CpG. The unchanged CpG (the eighth) is in bold. ApaI restriction sites are at the 5' and 3' ends when the sequence was cloned into the FIX expression cassette. SEQ ID NO:41 is CpG9-ApoE/hAAT with these restriction enzyme sites (underlined).

gggcccatgccacctccaacatccactgacccttggaatttggtggagagga
gcagaggttgtcctggtgtggtttaggtagtgtgagaggggtacctggggatctt
gctaccagtggaacagccactaaggattctgcagtgagagcagagggccagctaa
gtggtactctcccagagactgtctgactcatgccacccccctccaccttggacaca
ggatgctgtggtttctgagccaggtacaatgactcctttcagtaagtgcagtgga
agctgtacactgcccaggcaaagcgtctgggcagtgtaggtgggtgactcagatc
ccagccagtggacttagcccctgtttgctcctctgataactggggtgaccttggt
taatattcaccagcagcctccccctgttgcccctctggatccactgcttaaatatg
gatgaggacagggccc

Nucleic acid sequence of CpG10-ApoE/hAAT (SEQ ID NO:42). Every C changed to T, except leave ninth SpG with no change, and G changed to A in the seventh CpG. The unchanged CpG (the ninth) is in bold. ApaI restriction sites are at the 5' and 3' ends when the sequence was cloned into the FIX expression cassette. SEQ ID NO:43 is CpG10-ApoE/hAAT with these restriction enzyme sites (underlined).

gggcccatgccacctccaacatccactgacccctggaatttggtggagagga
gcagaggttgtcctggtgtggtttaggtagtgtgagaggggtacctggggatctt
gctaccagtggaacagccactaaggattctgcagtgagagcagagggccagctaa
gtggtactctcccagagactgtctgactcatgccacccccctccaccttggacaca
ggatgctgtggtttctgagccaggtacaatgactcctttcagtaagtgcagtgga
agctgtacactgcccaggcaaagtgtc**cg**ggcagtgtaggtgggtgactcagatc
ccagccagtggacttagcccctgtttgctcctctgataactggggtgaccttggt
taatattcaccagcagcctcccctgttgcccctctggatccactgcttaaatatg
gatgaggacagggccc

Nucleic acid sequence of CpG11-ApoE/hAAT (SEQ ID NO:44). Every C changed to T, except leave tenth CpG with no change, and G changed to A in the seventh CpG. The unchanged CpG (the tenth) is in bold. ApaI restriction sites are at the 5' and 3' ends when the sequence was cloned into the FIX expression cassette. SEQ ID NO:45 is CpG11-ApoE/hAAT with these restriction enzyme sites (underlined).

gggcccatgccacctccaacatccactgacccctggaatttggtggagagga
gcagaggttgtcctggtgtggtttaggtagtgtgagaggggtacctggggatctt
gctaccagtggaacagccactaaggattctgcagtgagagcagagggccagctaa
gtggtactctcccagagactgtctgactcatgccacccccctccaccttggacaca
ggatgctgtggtttctgagccaggtacaatgactcctttcagtaagtgcagtgga
agctgtacactgcccaggcaaagtgtctgggcag**cg**taggtgggtgactcagatc
ccagccagtggacttagcccctgtttgctcctctgataactggggtgaccttggt
taatattcaccagcagcctcccctgttgcccctctggatccactgcttaaatatg
gatgaggacagggccc

Nucleic acid sequence of CpG12-ApoE/hAAT (SEQ ID NO:46). Every C changed to T, except leave eleventh CpG with no change, and G changed to A in the seventh CpG. The unchanged CpG (the eleventh) is in bold. ApaI restriction sites are at the 5' and 3' ends when the sequence was cloned into the FIX expression cassette. SEQ ID NO:47 is CpG12-ApoE/hAAT with these restriction enzyme sites (underlined).

gggcccatgccacctccaacatccactgacccctggaatttggtggagagga
gcagaggttgtcctggtgtggtttaggtagtgtgagaggggtacctggggatctt
gctaccagtggaacagccactaaggattctgcagtgagagcagagggccagctaa
gtggtactctcccagagactgtctgactcatgccacccccctccaccttggacaca
ggatgctgtggtttctgagccaggtacaatgactcctttcagtaagtgcagtgga
agctgtacactgcccaggcaaagtgtctgggcagtgtagg**cg**ggtgactcagatc
ccagccagtggacttagcccctgtttgctcctctgataactggggtgaccttggt
taatattcaccagcagcctcccctgttgcccctctggatccactgcttaaatatg
gatgaggacagggccc

Nucleic acid sequence of CpG13-ApoE/hAAT (SEQ ID NO:48). Every C changed to T, except leave twelfth CpG with no change, and G changed to A in the seventh CpG. The unchanged CpG (the twelfth) is in bold. ApaI restriction sites are at the 5' and 3' ends when the sequence was cloned into the FIX expression cassette. SEQ ID NO:49 is CpG13-ApoE/hAAT with these restriction enzyme sites (underlined).

gggcccatgccacctccaacatccacttgacccctttggaatttttggtggagagga
gcagaggttgtcctggtgtggtttaggtagtgtgagaggggtacctgggatctt
gctaccagtggaacagccactaaggattctgcagtgagagcagagggccagctaa
gtggtactctcccagagactgtctgactcatgccacccccctccaccttggacaca
ggatgctgtggtttctgagccaggtacaatgactcctttcagtaagtgcagtgga
agctgtacactgcccaggcaaagtgtctgggcagtgtaggtgggcgactcagatc
ccagccagtggacttagcccctgtttgctcctctgataactggggtgaccttggt
taatattcaccagcagcctcccctgttgcccctctggatccactgcttaaatatg
gatgaggacagggccc

Nucleic acid sequence of CpG14-ApoE/hAAT (SEQ ID NO:50). Every C changed to T, except leave thirteenth CpG with no change, and G changed to A in the seventh CpG. The unchanged CpG (the thirteenth) is in bold. ApaI restriction sites are at the 5' and 3' ends when the sequence was cloned into the FIX expression cassette. SEQ ID NO:51 is CpG14-ApoE/hAAT with these restriction enzyme sites (underlined).

gggcccatgccacctccaacatccacttgacccctttggaatttttggtggagagga
gcagaggttgtcctggtgtggtttaggtagtgtgagaggggtacctgggatctt
gctaccagtggaacagccactaaggattctgcagtgagagcagagggccagctaa
gtggtactctcccagagactgtctgactcatgccacccccctccaccttggacaca
ggatgctgtggtttctgagccaggtacaatgactcctttcagtaagtgcagtgga
agctgtacactgcccaggcaaagtgtctgggcagtgtaggtgggtgactcagatc
ccagccagtggacttagcccctgtttgctcctccgataactggggtgaccttggt
taatattcaccagcagcctcccctgttgcccctctggatccactgcttaaatatg
gatgaggacagggccc

Nucleic acid sequence of CpG15-ApoE/hAAT (SEQ ID NO:52). Every C changed to T, except leave fourteenth CpG with no change, and G changed to A in the seventh CpG. The unchanged CpG (the fourteenth) is in bold. ApaI restriction sites are at the 5' and 3' ends when the sequence was cloned into the FIX expression cassette. SEQ ID NO:53 is CpG15-ApoE/hAAT with these restriction enzyme sites (underlined).

gggcccatgccacctccaacatccacttgacccctttggaatttttggtggagagga
gcagaggttgtcctggtgtggtttaggtagtgtgagaggggtacctgggatctt
gctaccagtggaacagccactaaggattctgcagtgagagcagagggccagctaa
gtggtactctcccagagactgtctgactcatgccacccccctccaccttggacaca
ggatgctgtggtttctgagccaggtacaatgactcctttcagtaagtgcagtgga
agctgtacactgcccaggcaaagtgtctgggcagtgtaggtgggtgactcagatc
ccagccagtggacttagcccctgtttgctcctctgataactggggtgaccttggt
taatattcaccagcagcctccccgttgcccctctggatccactgcttaaatatg
gatgaggacagggccc

Nucleic acid sequence of CpG16-ApoE/hAAT (SEQ ID NO:54). Every C changed to T, except leave fifteenth CpG with no change, and G changed to A in the seventh CpG. The unchanged CpG (the fifteenth) is in bold. ApaI restriction sites are at the 5' and 3' ends when the sequence was cloned into the FIX expression cassette. SEQ ID NO:55 is CpG16-ApoE/hAAT with these restriction enzyme sites (underlined).

gggcccatgccacctccaacatccactgacccctggaatttggtggagagga
gcagaggttgtcctggtgtggtttaggtagtgtgagaggggtacctggggatctt
gctaccagtggaacagccactaaggattctgcagtgagagcagagggccagctaa
gtggtactctcccagagactgtctgactcatgccacccccctccaccttggacaca
ggatgctgtggtttctgagccaggtacaatgactcctttcagtaagtgcagtgga
agctgtacactgcccaggcaaagtgtctgggcagtgtaggtgggtgactcagatc
ccagccagtggacttagcccctgtttgctcctctgataactggggtgaccttggt
taatattcaccagcagcctcccctgttgcccctctggatccactgcttaaataacg
gatgaggacagggccc

Nucleic acid sequence of CpG17-ApoE/hAAT (SEQ ID NO:56). Every C changed to T, except leave the sixteenth CpG with no change, and G changed to A in the seventh CpG. The unchanged CpG (the sixteenth) is in bold. ApaI restriction sites are at the 5' and 3' ends when the sequence was cloned into the FIX expression cassette. SEQ ID NO:57 is CpG17-ApoE/hAAT with these restriction enzyme sites (underlined).

gggcccatgccacctccaacatccactgacccctggaatttggtggagagga
gcagaggttgtcctggtgtggtttaggtagtgtgagaggggtacctggggatctt
gctaccagtggaacagccactaaggattctgcagtgagagcagagggccagctaa
gtggtactctcccagagactgtctgactcatgccacccccctccaccttggacaca
ggatgctgtggtttctgagccaggtacaatgactcctttcagtaagtgcagtgga
agctgtacactgcccaggcaaagtgtctgggcagtgtaggtgggtgactcagatc
ccagccagtggacttagcccctgtttgctcctctgataactggggtgaccttggt
taatattcaccagcagcctcccctgttgcccctctggatccactgcttaaatatg
gacgaggacagggccc

Nucleic acid sequence of CpG18-ApoE/hAAT (SEQ ID NO:58). The C of the fifth, seventh, eighth, tenth and eleventh CpG is removed, and C is changed to T for the remaining CpGs. ApaI restriction sites are at the 5' and 3' ends when the sequence was cloned into the FIX expression cassette. SEQ ID NO:59 is CpG18-ApoE/hAAT with these restriction enzyme sites (underlined).

gggcccatgccacctccaacatccactgacccctggaatttggtggagagga
gcagaggttgtcctggtgtggtttaggtagtgtgagaggggtacctggggatctt
gctaccagtggaacagccactaaggattctgcagtgagagcagagggccagctaa
gtggtactctcccagagactgtctgactcagccacccccctccaccttggacacag
gatgctgtggtttctgagccaggtacaatgactcctttggtaagtgcagtggaag
ctgtacactgcccaggcaaaggtctgggcaggtagggggtgactcagatcccagc
cagtggacttagcccctgtttgctcctctgataactggggtgaccttggttaata
ttcaccagcagcctcccctgttgcccctctggatccactgcttaaatatggatga
ggacagggccc

Nucleic acid sequence of CpG19-ApoE/hAAT (SEQ ID NO:60). C changed to T in the first through fifth and eighth through eleventh CpG, and the C of the seventh CpG is removed. All remaining CpGs (sixth and twelfth through sixteenth) are unchanged (bold). ApaI restriction sites are at the 5' and 3' ends when the sequence was cloned into the FIX

expression cassette. SEQ ID NO:61 is CpG19-ApoE/hAAT with these restriction enzyme sites (underlined).

gggcccatgccacctccaacatccacttgacccctttggaatttttggtggagagga
gcagaggttgtcctggtgtggtttaggtagtgtgagaggggtacctggggatctt
gctaccagtggaacagccactaaggattctgcagtgagagcagagggccagctaa
gtggtactctcccagagactgtctgactcatgccacccccctccaccttggacaca
ggacgctgtggtttctgagccaggtacaatgactcctttggtaagtgcagtggaa
gctgtacactgcccaggcaaagtgtctgggcagtgtaggtgggcgactcagatcc
cagccagtggacttagcccctgtttgctcctccgataactggggtgaccttggtt
aatattcaccagcagcctcccccgttgcccctctggatccactgcttaaatacgg
acgaggacagggccc

Nucleic acid sequence of CpG20-ApoE/hAAT (SEQ ID NO:62). Result of multiple deletions of regions without putative transcription factor binding sites. G changed to A in the only remaining CpG (the seventh CpG of SEQ ID NO:22). ApaI restriction sites are at the 5' and 3' ends when the sequence was cloned into the FIX expression cassette. SEQ ID NO:63 is CpG20-ApoE/hAAT with these restriction enzyme sites (underlined).

gggcccatgccacctccaaggagcagaggtgatcttgctaccagtggagagcaga
gggccagctctcccagagactgtctgactcagccacccctccaccttggacaca
ggaggtttctgagccatcctttcagtaagtgcatgtacactgcccagctgggcag
ctcagatcccagccagtggacttagcccctgtttgctcctctgataactggggtg
accttggttaatattcaccagcagcctgttgcccctctggatccactgcttaaag
ggccc

Nucleic acid sequence of CpG21-ApoE/hAAT (SEQ ID NO:64). C changed to T in all CpGs, except the fifth and sixth CpGs, where G is changed to C and A, respectively. ApaI restriction sites are at the 5' and 3' ends when the sequence was cloned into the FIX expression cassette. SEQ ID NO:65 is CpG21-ApoE/hAAT with these restriction enzyme sites (underlined).

gggcccatgccacctccaacatccacttgacccctttggaatttttggtggagagga
gcagaggttgtcctggtgtggtttaggtagtgtgagaggggtacctggggatctt
gctaccagtggaacagccactaaggattctgcagtgagagcagagggccagctaa
gtggtactctcccagagactgtctgactcaccccaccccctccaccttggacaca
ggacactgtggtttctgagccaggtacaatgactcctttttggtaagtgcagtgga
agctgtacactgcccaggcaaagtgtctgggcagtgtaggtgggtgactcagatc
ccagccagtggacttagcccctgtttgctcctctgataactggggtgaccttggt
taatattcaccagcagcctcccctgttgcccctctggatccactgcttaaatatgg
atgaggacagggccc

Nucleic acid sequence of CpG22-ApoE/hAAT (SEQ ID NO:66). C changed to T in all CpGs, except leave fifth CpG with no change (bold), and change G to A in the sixth CpG. ApaI restriction sites are at the 5' and 3' ends when the sequence was

cloned into the FIX expression cassette. SEQ ID NO:67 is CpG22-ApoE/hAAT with these restriction enzyme sites (underlined).

gggcccatgccacctccaacatccacttgacccccttggaatttttggtggagagga
gcagaggttgtcctggtgtggtttaggtagtgtgagaggggtacctggggatctt
gctaccagtggaacagccactaaggattctgcagtgagagcagagggccagctaa
gtggtactctcccagagactgtctgactcacgccaccccctccaccttggacaca
ggacactgtggtttctgagccaggtacaatgactccttttggtaagtgcagtgga
agctgtacactgcccaggcaaagtgtctgggcagtgtaggtgggtgactcagatc
ccagccagtggacttagcccctgtttgctcctctgataactggggtgaccttggt
taatattcaccagcagcctcccctgttgcccctctggatccactgcttaaatatg
gatgaggacagggccc

Amino acid sequence of FVIII-BDD (SQ sequence bold/underlined) (SEQ ID NO:68).
MQIELSTCFFLCLLRFCFSATRRYYLGAVELSWDYMQSDLGELPVDARFPPRVPKSFPFNTS
VVYKKTLFVEFTDHLFNIAKPRPPWMGLLGPTIQAEVYDTVVITLKNMASHPVSLHAVGVSY
WKASEGAEYDDQTSQREKEDDKVFPGGSHTYVWQVLKENGPMASDPLCLTYSYLSHVDLVKD
LNSGLIGALLVCREGSLAKEKTQTLHKFILLFAVFDEGKSWHSETKNSLMQDRDAASARAWP
KMHTVNGYVNRSLPGLIGCHRKSVYWHVIGMGTTPEVHSIFLEGHTFLVRNHRQASLEISPI
TFLTAQTLLMDLGQFLLFCHISSHQHDGMEAYVKVDSCPEEPQLRMKNNEEAEDYDDDLTDS
EMDVVRFDDDNSPSFIQIRSVAKKHPKTWVHYIAAEEEDWDYAPLVLAPDDRSYKSQYLNNG
PQRIGRKYKKVRFMAYTDETFKTREAIQHESGILGPLLYGEVGDTLLIIFKNQASRPYNIYP
HGITDVRPLYSRRLPKGVKHLKDFPILPGEIFKYKWTVTVEDGPTKSDPRCLTRYYSSFVNM
ERDLASGLIGPLLICYKESVDQRGNQIMSDKRNVILFSVFDENRSWYLTENIQRFLPNPAGV
QLEDPEFQASNIMHSINGYVFDSLQLSVCLHEVAYWYILSIGAQTDFLSVFFSGYTFKHKMV
YEDTLTLFPFSGETVFMSMENPGLWILGCHNSDFRNRGMTALLKVSSCDKNTGDYYEDSYED
ISAYLLSKNNAIEPR**SFSQNPPVLKRHQR**EITRTTLQSDQEEIDYDDTISVEMKKEDFDIYD
EDENQSPRSFQKKTRHYFIAAVERLWDYGMSSSPHVLRNRAQSGSVPQFKKVVFQEFTDGSF
TQPLYRGELNEHLGLLGPYIRAEVEDNIMVTFRNQASRPYSFYSSLISYEEDQRQGAEPRKN
FVKPNETKTYFWKVQHHMAPTKDEFDCKAWAYFSDVDLEKDVHSGLIGPLLVCHTNTLNPAH
GRQVTVQEFALFFTIFDETKSWYFTENMERNCRAPCNIQMEDPTFKENYRFHAINGYIMDTL
PGLVMAQDQRIRWYLLSMGSNENIHSIHFSGHVFTVRKKEEYKMALYNLYPGVFETVEMLPS
KAGIWRVECLIGEHLHAGMSTLFLVYSNKCQTPLGMASGHIRDFQITASGQYGQWAPKLARL
HYSGSINAWSTKEPFSWIKVDLLAPMIIHGIKTQGARQKFSSLYISQFIIMYSLDGKKWQTY
RGNSTGTLMVFFGNVDSSGIKHNIFNPPIIARYIRLHPTHYSIRSTLRMELMGCDLNSCSMP
LGMESKAISDAQITASSYFTNMFATWSPSKARLHLQGRSNAWRPQVNNPKEWLQVDFQKTMK
VTGVTTQGVKSLLTSMYVKEFLISSSQDGHQWTLFFQNGKVKVFQGNQDSFTPVVNSLDPPL
LTRYLRIHPQSWVHQIALRMEVLGCEAQDLY

SQ sequence (SEQ ID NO:69).
SFSQNPPVLKRHQR

Wild-type FVIII-BDD cDNA (SEQ ID NO:70).
atgcaaatagagctctccacctgcttctttctgtgcttttgcgattctgctttagtgccac
cagaagatactacctgggtgcagtggaactgtcatgggactatatgcaaagtgatctcggtg
agctgcctgtggacgcaagatttcctcctagagtgccaaaatcttttccattcaacacctca
gtcgtgtacaaaaagactctgtttgtagaattcacggatcacctttcaacatcgctaagcc

aaggccaccctggatgggtctgctaggtcctaccatccaggctgaggtttatgatacagtgg
tcattacacttaagaacatggcttcccatcctgtcagtcttcatgctgttggtgtatcctac
tggaaagcttctgagggagctgaatatgatgatcagaccagtcaaagggagaaagaagatga
taaagtcttccctggtggaagccatacatatgtctggcaggtcctgaaagagaatggtccaa
tggcctctgacccactgtgccttacctactcatatctttctcatgtggacctggtaaaagac
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gacacagaccttgcacaaatttatactacttttgctgtatttgatgaagggaaaagttggc
actcagaaacaaagaactccttgatgcaggatagggatgctgcatctgctcgggcctggcct
aaaatgcacacagtcaatggttatgtaaacaggtctctgccaggtctgattggatgccacag
gaaatcagtctattggcatgtgattggaatgggcaccactcctgaagtgcactcaatattcc
tcgaaggtcacacatttcttgtgaggaaccatcgccaggcgtccttggaaatctcgccaata
actttccttactgctcaaacactcttgatggaccttggacagtttctactgttttgtcatat
ctcttcccaccaacatgatggcatggaagcttatgtcaaagtagacagctgtccagaggaac
cccaactacgaatgaaaaataatgaagaagcggaagactatgatgatgatcttactgattct
gaaatggatgtggtcaggtttgatgatgacaactctccttcctttatccaaattcgctcagt
tgccaagaagcatcctaaaacttgggtacattacattgctgctgaagaggaggactgggact
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cctcagcggattggtaggaagtacaaaaaagtccgatttatggcatacacagatgaaaccctt
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cacggaatcactgatgtccgtcctttgtattcaaggagattaccaaaaggtgtaaaacattt
gaaggattttccaattctgccaggagaaatattcaaatataaatggacagtgactgtagaag
atgggccaactaaatcagatcctcggtgcctgacccgctattactctagtttcgttaatatg
gagagagatctagcttcaggactcattggccctctcctcatctgctacaaagaatctgtaga
tcaaagaggaaaccagataatgtcagacaagaggaatgtcatcctgttttctgtatttgatg
agaaccgaagctggtacctcacagagaatatacaacgctttctccccaatccagctggagtg
cagcttgaggatccagagttccaagcctccaacatcatgcacagcatcaatggctatgtttt
tgatagtttgcagttgtcagtttgtttgcatgaggtggcatactggtacattctaagcattg
gagcacagactgacttcctttctgtcttcttctctggatataccttcaaacacaaaatggtc
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cccaggtctatggattctggggtgccacaactcagactttcggaacagaggcatgaccgcct
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atttcagcatacttgctgagtaaaaacaatgccattgaaccaagaagcttctcccaaaaccc
accagtcttgaaacgccatcaacgggaaataactcgtactactcttcagtcagatcaagagg
aaattgactatgatgataccatatcagttgaaatgaagaaggaagattttgacatttatgat
gaggatgaaaatcagagcccccgcagctttcaaaagaaaacacgacactattttattgctgc
agtggagaggctctgggattatgggatgagtagctccccacatgttctaagaaacagggctc
agagtggcagtgtccctcagttcaagaaagttgttttccaggaatttactgatggctccttt
actcagcccttataccgtggagaactaaatgaacatttgggactcctggggccatatataag
agcagaagttgaagataatatcatggtaactttcagaaatcaggcctctcgtccctattcct
tctattctagccttatttcttatgaggaagatcagaggcaaggagcagaacctagaaaaaac
tttgtcaagcctaatgaaaccaaaacttacttttggaaagtgcaacatcatatggcacccac
taaagatgagtttgactgcaaagcctgggcttatttctctgatgttgacctggaaaaagatg
tgcactcaggcctgattggacccccttctggtctgccacactaacacactgaaccctgctcat
gggagacaagtgacagtacaggaatttgctctgtttttcaccatctttgatgagaccaaaag
ctggtacttcactgaaaatatggaaagaaactgcagggctcccctgcaatatccagatggaag
atcccacttttaaagagaattatcgcttccatgcaatcaatggctacataatggatacacta
cctggcttagtaatggctcaggatcaaaggattcgatggtatctgctcagcatgggcagcaa
tgaaaacatccattctattcatttcagtggacatgtgttcaccgtacgaaaaaaagaggagt
ataaaatggcactgtacaatctctatccaggtgtttttgagacagtggaaatgttaccatcc

aaagctggaatttggcgggtggaatgccttattggcgagcatctacatgctgggatgagcac
acttttctggtgtacagcaataagtgtcagactcccctgggaatggcttctggacacatta
gagattttcagattacagcttcaggacaatatggacagtgggccccaaagctggccagactt
cattattccggatcaatcaatgcctggagcaccaaggagccctttttcttggatcaaggtgga
tctgttggcaccaatgattattcacggcatcaagacccagggtgcccgtcagaagttctcca
gcctctacatctctcagtttatcatcatgtatagtcttgatgggaagaagtggcagacttat
cgaggaaattccactggaaccttaatggtcttctttggcaatgtggattcatctgggataaa
acacaatatttttaaccctccaattattgctcgatacatccgtttgcacccaactcattata
gcattcgcagcactcttcgcatggagttgatgggctgtgatttaaatagttgcagcatgcca
ttgggaatggagagtaaagcaatatcagatgcacagattactgcttcatcctactttaccaa
tatgtttgccacctggtctccttcaaaagctcgacttcacctccaagggaggagtaatgcct
ggagacctcaggtgaataatccaaaagagtggctgcaagtggacttccagaagacaatgaaa
gtcacaggagtaactactcagggagtaaaatctctgcttaccagcatgtatgtgaaggagtt
cctcatctccagcagtcaagatggccatcagtggactctctttttcagaatggcaaagtaa
aggtttttcagggaaatcaagactccttcacacctgtggtgaactctctagacccaccgtta
ctgactcgctaccttcgaattcacccccagagttgggtgcaccagattgccctgaggatgga
ggttctgggctgcgaggcacaggacctctactga

FVIII-BDD encoding CpG reduced nucleic acid variant (SEQ ID NO:71)

atgcagattgagctgtctacctgcttcttcctgtgcctgctgaggttctgcttctctgct
accaggaggtactacctgggggctgtggagctgagctgggattacatgcagtctgacctg
ggggagctgcctgtggatgccaggtttccccccagggtgcccaagagcttccccttcaat
acctctgtggtgtataagaagaccctgtttgtggagttcactgatcatctgttcaacatt
gctaaacccaggcccccctggatggggctgctgggccctaccatccaggctgaggtgtat
gacactgtggtgatcactctgaagaacatggctagccatcctgtgtctctgcatgctgtg
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gagaaggaggatgacaaggtgttccctgggggctctcacacctatgtctggcaggtgctg
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aacaggagctggtacctgactgagaacattcagaggtttctgcccaaccctgctggggtg

cagctggaggaccctgaattccaggcctctaacatcatgcacagcattaatggctatgtg
tttgacagcctgcagctgtctgtgtgcctgcatgaggtggcctactggtacattctgagc
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cagtctgaccaggaggagattgactatgatgacaccatctctgtggagatgaagaaggag
gattttgatatttatgatgaggatgaaaaccagagccccaggagctttcagaagaagact
aggcactatttcattgctgctgtggagaggctgtgggactatggcatgtcttctagcccc
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Nucleic acid sequence of intron in AAV-WINT (SEQ ID NO:93)
AGGTAAGTGCCGTGTGTGGTTCCCGCGGGCCTGGCCTCTTTACGGGTTATGGCCCTTGCGTG
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FIX (SEQ ID NO:94)
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WHAT IS CLAIMED IS:

1. An expression cassette comprising a nucleic acid sequence encoding a Factor VIII protein having a B domain deletion (FVIII-BDD), wherein said expression cassette comprises a sequence at least 98% identical to the sequence of SEQ ID NO:1.

2. The expression cassette of claim 1, wherein said expression cassette comprises a sequence at least 99% identical to the sequence of SEQ ID NO:1.

3. The expression cassette of claim 1, wherein said expression cassette comprises the sequence of SEQ ID NO:1.

4. The expression cassette of claim 1, wherein said expression cassette consists of the sequence of SEQ ID NO:1.

5. An expression cassette comprising a regulatory element operably linked to a nucleic acid sequence encoding a Factor VIII protein having a B domain deletion (FVIII-BDD), wherein no intron is present between said regulatory element and said nucleic acid sequence, and wherein said expression cassette comprises a sequence at least 91% identical to SEQ ID NO:1.

6. An expression cassette comprising
   a. a regulatory element at least 90% identical to the sequence of any of SEQ ID NOs:2-67, and
   b. a nucleic acid sequence encoding a Factor VIII protein having a B domain deletion (FVIII-BDD), said nucleic acid sequence having at least 90% identity to the sequence of SEQ ID NO:77,

   wherein said regulatory element is operably linked to said nucleic acid sequence, and wherein no intron is present between said regulatory element and said nucleic acid sequence.

7. An expression cassette comprising
   a. a regulatory element at least 90% identical to the sequence of any of SEQ ID NOs:2-67, and

b. a nucleic acid sequence encoding a Factor VIII protein having a B domain deletion (FVIII-BDD), said nucleic acid sequence having at least 90% identity to the sequence of SEQ ID NO:77,

wherein said regulatory element is operably linked to said nucleic acid sequence, and wherein no more than 0 – 5, 5 – 10, 10 – 15, 15 – 20, 20 – 25, 25 – 30, 30 – 35, 35 – 40, 40 – 45, 45 – 50, 50 – 55, 55 – 60, 60 – 65, 65 – 70, 70 – 75, 75 – 80, 80 – 85, 85 – 90, 90 – 95, 95 – 100, 100 – 105, 106 or 107 nucleotides of untranslated nucleic acid is between said regulatory element and said nucleic acid sequence.

8. The expression cassette of any one of claims 5 – 7, wherein said regulatory element comprises a nucleotide sequence at least 95% identical to any of SEQ ID NOs:2-67.

9. The expression cassette of any one of claims 5 – 8, wherein said regulatory element has the same total number of reduced CpGs as set forth in the sequence of any of SEQ ID NOs:4-21 or 24-67.

10. The expression cassette of any one of claims 5 – 9, wherein said regulatory element comprises the sequence of any of SEQ ID NOs:2-21 or 24-67 having CpG(s) substituted to be CpT, CpA, TpG, or ApG at the same position(s) as set forth in the sequence of any of SEQ ID NOs:4-21 or 24-67.

11. The expression cassette of any one of claims 5 – 10, wherein said nucleic acid sequence exhibits greater expression when compared to expression from an expression cassette having (a) an intron, or (b) 108 or more nucleotides of untranslated nucleic acid, between said regulatory element and said nucleic acid sequence.

12. The expression cassette of any of one claims 5 – 11, wherein said encoded FVIII-BDD exhibits greater biological activity as compared to expression from an expression cassette having (a) an intron, or (b) 108 or more nucleotides of untranslated nucleic acid, between said regulatory element and said nucleic acid sequence.

13. The expression cassette of claim 12, wherein biological activity is determined by a clotting assay or reduced bleeding in a FVIII assay or FVIII deficiency model.

14. The expression cassette of any one of claims 5 – 13, wherein said expression cassette is more efficiently packaged into an AAV vector when compared to packaging of an expression cassette having (a) an intron, or (b) 108 or more nucleotides of untranslated nucleic acid, between said regulatory element and said nucleic acid sequence.

15. A nucleic acid sequence, comprising SEQ ID NO:2, 3, 22 or 23 modified to have fewer cytosine-guanine dinucleotides (CpGs).

16. The nucleic acid sequence of claim 15, comprising SEQ ID NO:2, 3, 22 or 23 modified to have 1 fewer CpG.

17. The nucleic acid sequence of claim 15, comprising SEQ ID NO:2, 3, 22 or 23 modified to have 2 fewer CpGs.

18. The nucleic acid sequence of claim 15, comprising SEQ ID NO:2, 3, 22 or 23 modified to have 3 fewer CpGs.

19. The nucleic acid sequence of claim 15, comprising SEQ ID NO:2, 3, 22 or 23 modified to have 4 fewer CpGs.

20. The nucleic acid sequence of claim 15, comprising SEQ ID NO:22 or 23 modified to have 5 fewer CpGs.

21. The nucleic acid sequence of claim 15, comprising SEQ ID NO:22 or 23 modified to have 6 fewer CpGs.

22. The nucleic acid sequence of claim 15, comprising SEQ ID NO:22 or 23 modified to have 7 fewer CpGs.

23. The nucleic acid sequence of claim 15, comprising SEQ ID NO:22 or 23 modified to have 8 fewer CpGs.

24. The nucleic acid sequence of claim 15, comprising SEQ ID NO:22 or 23 modified to have 9 fewer CpGs.

25. The nucleic acid sequence of claim 15, comprising SEQ ID NO:22 or 23 modified to have 10 fewer CpGs.

26. The nucleic acid sequence of claim 15, comprising SEQ ID NO:22 or 23 modified to have 11 fewer CpGs.

27. The nucleic acid sequence of claim 15, comprising SEQ ID NO:22 or 23 modified to have 12 fewer CpGs.

28. The nucleic acid sequence of claim 15, comprising SEQ ID NO:22 or 23 modified to have 13 fewer CpGs.

29. The nucleic acid sequence of claim 15, comprising SEQ ID NO:22 or 23modified to have 14 fewer CpGs.

30. The nucleic acid sequence of claim 15, comprising SEQ ID NO:22 or 23 modified to have 15 fewer CpGs.

31. The nucleic acid sequence of claim 15, comprising SEQ ID NO:22 or 23 modified to have 16 fewer CpGs.

32. The nucleic acid sequence of claim 15, comprising SEQ ID NO:2, 3, 22 or 23 modified to have 1 or 0 CpGs.

33. The nucleic acid sequence of claim 15, comprising SEQ ID NO:2, 3, 22 or 23 modified to have 0 CpGs.

34. The nucleic acid sequence of any of claims 15 – 33, wherein at least the first CpG from the 5' end in SEQ ID NO:2, 3, 22 or 23 is modified to not be CpG.

35. The nucleic acid sequence of any of claims 15 – 33, wherein at least the second CpG from the 5' end in SEQ ID NO:2, 3, 22 or 23 is modified to not be CpG.

36. The nucleic acid sequence of any of claims 15 – 33, wherein at least the third CpG from the 5' end in SEQ ID NO:2, 3, 22 or 23 is modified to not be CpG.

37. The nucleic acid sequence of any of claims 15 – 33, wherein at least the fourth CpG from the 5' end in SEQ ID NO:2, 3, 22 or 23 is modified to not be CpG.

38. The nucleic acid sequence of any of claims 15 – 33, wherein at least the fifth CpG from the 5' end in SEQ ID NO:22 or 23 is modified to not be CpG.

39. The nucleic acid sequence of any of claims 15 – 33, wherein at least the sixth CpG from the 5' end in SEQ ID NO:22 or 23 is modified to not be CpG.

40. The nucleic acid sequence of any of claims 15 – 33, wherein at least the seventh CpG from the 5' end in SEQ ID NO:22 or 23 is modified to not be CpG.

41. The nucleic acid sequence of any of claims 15 – 33, wherein at least the eighth CpG from the 5' end in SEQ ID NO:22 or 23 is modified to not be CpG.

42. The nucleic acid sequence of any of claims 15 – 33, wherein at least the ninth CpG from the 5' end in SEQ ID NO:22 or 23 is modified to not be CpG.

43. The nucleic acid sequence of any of claims 15 – 33, wherein at least the 10^{th} CpG from the 5' end in SEQ ID NO:22 or 23 is modified to not be CpG.

44. The nucleic acid sequence of any of claims 15 – 33, wherein at least the 11^{th} CpG from the 5' end in SEQ ID NO:22 or 23 is modified to not be CpG.

45. The nucleic acid sequence of any of claims 15 – 33, wherein at least the 12^{th} CpG from the 5' end in SEQ ID NO:22 or 23 is modified to not be CpG.

46. The nucleic acid sequence of any of claims 15 – 33, wherein at least the 13^{th} CpG from the 5' end in SEQ ID NO:22 or 23 is modified to not be CpG.

47. The nucleic acid sequence of any of claims 15 – 33, wherein at least the 14^{th} CpG from the 5' end in SEQ ID NO:22 or 23 is modified to not be CpG.

48. The nucleic acid sequence of any of claims 15 – 33, wherein at least the 15^{th} CpG from the 5' end in SEQ ID NO:22 or 23 is modified to not be CpG.

49. The nucleic acid sequence of any of claims 15 – 33, wherein at least the 16^{th} CpG from the 5' end in SEQ ID NO:22 or 23 is modified to not be CpG.

50. A nucleic acid sequence at least 95% identical to the sequence of the nucleic acid sequence of any of claims 15 – 49.

51. A polynucleotide comprising a nucleic acid sequence at least 95% identical to the sequence of any of SEQ ID NOs:4-21 or 24-67 with or without 5' and/or 3' flanking 5-7 nucleotides.

52. A polynucleotide comprising a nucleic acid sequence at least 95% identical to the sequence of any of SEQ ID NOs:4-21 or 24-67, and having the same number of reduced CpGs as set forth in any of SEQ ID NOs:4-21 or 24-67.

53. A polynucleotide comprising a nucleic acid sequence at least 95% identical to the sequence of any of SEQ ID NOs:4-21 or 24-67, and having reduced CpG(s) at the same position(s) as set forth in any of SEQ ID NOs:4-21 or 24-67.

54. The nucleic acid sequence or polynucleotide of any of claims 15 – 53, wherein said modified SEQ ID NO:2, 3, 22 or 23 has the G nucleotide in at least one CpG substituted with a T or A nucleotide to be a CpT or CpA dinucleotide, or has the C nucleotide in at least one CpG substituted with a T or A nucleotide to be a TpG or ApG dinucleotide.

55. The nucleic acid sequence or polynucleotide of any of claims 15 – 54, wherein the seventh CpG from the 5' end in said modified SEQ ID NO:22 or 23 does not have the G nucleotide substituted.

56. The nucleic acid sequence or polynucleotide of any of claims 15 – 54, wherein the seventh CpG from the 5' end in said modified SEQ ID NO:22 or 23 has the G nucleotide substituted with a T or A nucleotide to be a CpT or CpA dinucleotide.

57. The nucleic acid sequence or polynucleotide of any of claims 15 – 54, wherein the seventh CpG from the 5' end in said modified SEQ ID NO:22 or 23 has the G nucleotide substituted with a T or A nucleotide to be a CpT or CpA dinucleotide, and at least one additional CpG dinucleotide in said modified SEQ ID NO:22 or 23 has a G substituted with a T nucleotide to be a CpT dinucleotide in said modified SEQ ID NO:22 or 23.

58. The nucleic acid sequence or polynucleotide of any of claims 15 – 54, wherein the seventh CpG from the 5' end in said modified SEQ ID NO:22 or 23 has the G nucleotide substituted with a T or A nucleotide to be a CpT or CpA dinucleotide, and

at least one additional CpG dinucleotide in said modified SEQ ID NO:22 or 23 has a G substituted with a A nucleotide to be a CpA dinucleotide in said modified SEQ ID NO:22 or 23.

59. The nucleic acid sequence or polynucleotide of any of claims 15 – 54, wherein the seventh CpG from the 5' end in said modified SEQ ID NO:22 or 23 has the G nucleotide substituted with a T or A nucleotide to be a CpT or CpA dinucleotide, and at least two additional CpGs in said modified SEQ ID NO:22 or 23 has a G substituted with a T nucleotide to be a CpT dinucleotide in said modified SEQ ID NO:22 or 23.

60. The nucleic acid sequence or polynucleotide of any of claims 15 – 54, wherein the seventh CpG from the 5' end in said modified SEQ ID NO:22 or 23 has the G nucleotide substituted with a T or A nucleotide to be a CpT or CpA dinucleotide, and at least two additional CpGs in said modified SEQ ID NO:22 or 23 has a G substituted with a A nucleotide to be a CpA dinucleotide in said modified SEQ ID NO:22 or 23.

61. The nucleic acid sequence or polynucleotide of any of claims 15 – 60, wherein said modified SEQ ID NO:2, 3, 22 or 23 has the G and/or C nucleotide in at least one CpG deleted.

62. The nucleic acid sequence or polynucleotide of any of claims 15 – 61, operably linked to a transgene.

63. The nucleic acid sequence or polynucleotide of any of claims 15 – 61, wherein said nucleic acid sequence or polynucleotide confers transcription on an operably linked transgene that is within about 50% of the transcription conferred by unmodified SEQ ID NO:2, 3, 22 or 23.

64. The nucleic acid sequence or polynucleotide of any of claims 15 – 61, wherein said nucleic acid sequence or polynucleotide confers transcription on an operably linked transgene that is within about 25-50% of the transcription conferred by unmodified SEQ ID NO:2, 3, 22 or 23.

65. The nucleic acid sequence or polynucleotide of any of claims 15 – 61, wherein said nucleic acid sequence or polynucleotide confers transcription on an operably linked transgene that is within about 5-100% of the transcription conferred by unmodified SEQ ID NO:2, 3, 22 or 23.

66. An expression cassette comprising the nucleic acid sequence or polynucleotide of any of claims 15 – 61 and a transgene.

67. The expression cassette of claim 66, wherein said nucleic acid sequence or polynucleotide is positioned 5' of said transgene.

68. The expression cassette of claim 66 or 67, wherein said transgene encodes a therapeutic protein that is expressed in liver cells and secreted into the systemic circulation.

69. The expression cassette of claim 68, wherein said therapeutic protein treats or prevents a neurodegenerative or central nervous system (CNS) disease.

70. The expression cassette of claim 69, wherein said therapeutic protein is a protective ApoE isoform.

71. The expression cassette of claim 70, wherein said therapeutic protein is ApoE ε2 isoform.

72. The expression cassette of claim 68, wherein said therapeutic protein treats or prevents an autoimmune disease or allergic disease.

73. The expression cassette of claim 72, wherein said therapeutic protein is a fusion protein comprising an unwanted antigen and a leader sequence that drives secretion of said therapeutic protein from the cell.

74. The expression cassette of claim 73, wherein said unwanted antigen is the extracellular domain of myelin oligodendrocyte glycoprotein (MOG) or a fragment thereof.

75. The expression cassette of claim 68, wherein said therapeutic protein is a blood coagulation or clotting factor protein.

76. The expression cassette of claim 75, wherein said blood coagulation or clotting factor protein is Factor IX (FIX), Factor VIII (FVIII), Factor VII (FVII) or Protein C.

77. The expression cassette of claim 76, wherein said Factor VIII is encoded by a nucleic acid sequence at least 95% identical to the sequence of SEQ ID NO:68.

78. The expression cassette of claim 68, wherein said therapeutic protein is a lysosomal storage enzyme.

79. The expression cassette of claim 78, wherein said lysosomal storage enzyme is acid alpha-glucosidase (GAA) or alpha-galactosidase (GLA).

80. The expression cassette of claim 68, wherein the therapeutic protein treats of prevents an inflammatory disease or disorder.

81. The expression cassette of claim 68, wherein said therapeutic protein treats or prevents hereditary angioedema (HAE).

82. The expression cassette of claim 81, wherein said therapeutic protein is C1 esterase inhibitor (C1EI).

83. An adeno-associated virus (AAV) vector comprising the nucleic acid sequence, or polynucleotide or expression cassette of any of claims 1 – 82.

84. The AAV vector of claim 83, wherein said AAV vector comprises one or more of:
a) an AAV capsid; and

b) one or more AAV inverted terminal repeats (ITRs), wherein said AAV ITR(s) flanks the 5' or 3' terminus of said nucleic acid sequence, said polynucleotide, and/or said transgene.

85. The AAV vector of claim 84, further comprising an intron positioned within said flanking 5' or 3' ITR.

86. The AAV vector of claim 85, wherein at least one of said intron or one or more ITRs is modified to have reduced CpGs.

87. The AAV vector of any of claims 83 – 86, wherein said AAV capsid serotype comprises a modified or variant AAV VP1, VP2 and/or VP3 capsid having 90% or more sequence identity to AAV1, AAV2, AAV3, AAV3B, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, Rh10, Rh74 or AAV-2i8 VP1, VP2 and/or VP3 sequences, or a capsid having 95% or more sequence identity to AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, Rh10, Rh74, AAV-2i8, SEQ ID NO:91 or SEQ ID NO:92 VP1, VP2 and/or VP3 sequences, or a capsid having 100% sequence identity to AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, Rh10, Rh74, AAV-2i8, SEQ ID NO:91 or SEQ ID NO:92 VP1, VP2 and/or VP3 sequences.

88. The AAV vector of any of claims 83 – 87, wherein said ITRs comprise one or more ITRs of any of: AAV1, AAV2, AAV3, AAV3B, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, Rh10, or Rh74 AAV serotypes, or a combination thereof.

89. The AAV vector of any of claims 83 – 88, further comprising an ITR, a polyA signal and/or intron sequence.

90. A pharmaceutical composition comprising a plurality of AAV vectors of any of claims 83 – 89 in a biologically compatible carrier or excipient.

91. The pharmaceutical composition of claim 90, further comprising empty AAV capsids.

92. The pharmaceutical composition of claim 91, wherein the ratio of said empty AAV capsids to said AAV vector is within or between about 100:1-50:1, from about 50:1-25:1, from about 25:1-10:1, from about 10:1-1:1, from about 1:1-1:10, from about 1:10-1:25, from about 1:25-1:50, or from about 1:50-1:100.

93. The pharmaceutical composition of claim 91, wherein the ratio of said empty AAV capsids to said AAV vector is about 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, or 10:1.

94. The pharmaceutical composition of any of claims 90 – 93, further comprising a surfactant.

95. A method of treating a human in need of blood coagulation or clotting factor, comprising:
(a) providing the expression cassette of any of claims 1 – 14, 66- 68, 75 – 77, the AAV vector of any of claims 83 – 89, or the pharmaceutical composition of any of claims 90 – 94; and
(b) administering an amount of said expression cassette, polynucleotide, AAV vector, or pharmaceutical composition to said human, wherein said blood coagulation or clotting factor is expressed in said human.

96. The method of claim 95, wherein said human has hemophilia A or B.

97. The method of claim 95, wherein said AAV vector is administered to said human intravenously, intraarterially, intra-cavity, intramucosally, or via catheter.

98. The method of claim 95, wherein said blood coagulation or clotting factor is expressed at increased levels.

99. The method of claim 95, wherein said blood coagulation or clotting factor is expressed at greater than 1% of the levels of the blood coagulation or clotting factor found in a human not in need of blood coagulation or clotting factor.

100. The method of claim 95, wherein said blood coagulation or clotting factor is expressed at about 1%-40% of the levels of the blood coagulation or clotting factor found in a human not in need of blood coagulation or clotting factor.

101. The method of claim 95, wherein said blood coagulation or clotting factor is expressed at about 5%-30% of the levels of the blood coagulation or clotting factor found in a human not in need of blood coagulation or clotting factor.

102. The method of claim 95, wherein said AAV vector is administered in a range from about $1X10^{8}$ to about $1X10^{14}$ vector genomes per kilogram (vg/kg) of the weight of said human.

103. The method of claim 95, wherein said human has hemophilia A with inhibitory antibodies to Factor VIII (FVIII).

104. A method of treating hemophila A with inhibitory antibodies to Factor VIII (FVIII) in a human in need thereof, comprising:

(a) providing the expression cassette of any of claims 1 – 14, 66 - 68, 75 – 77, the AAV vector of any of claims 83 – 89, or the pharmaceutical composition of any of claims 90 – 94; and

(b) administering an amount of said nucleic acid, polynucleotide, expression cassette, AAV vector, or pharmaceutical composition to said human, wherein said blood coagulation or clotting factor is Factor VIII (FVIII) and is expressed in said human.

Figure 1

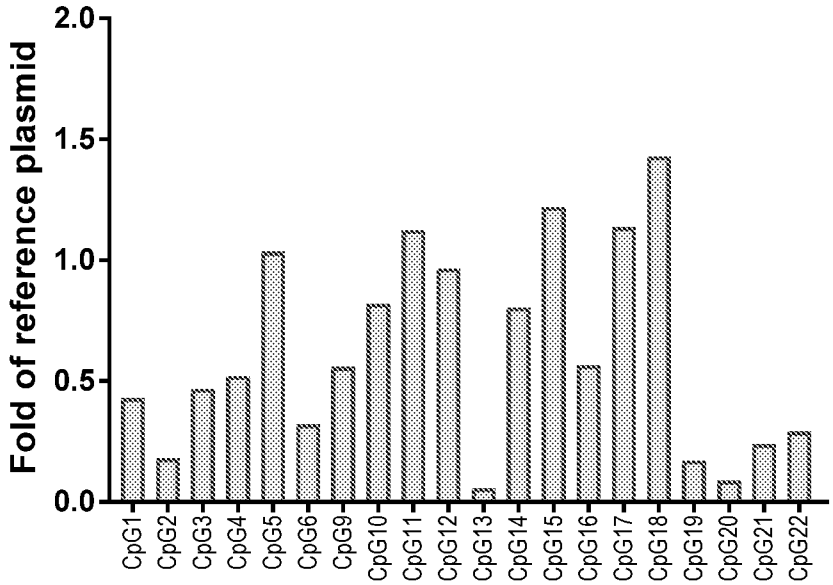


Figure 2

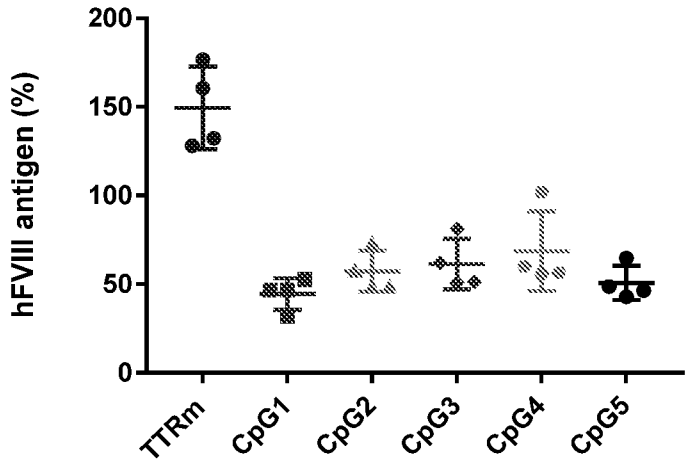


Figure 3

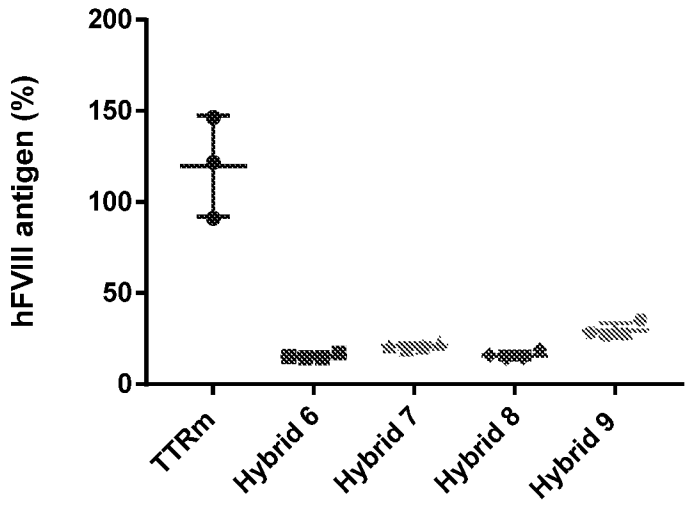


Figure 4

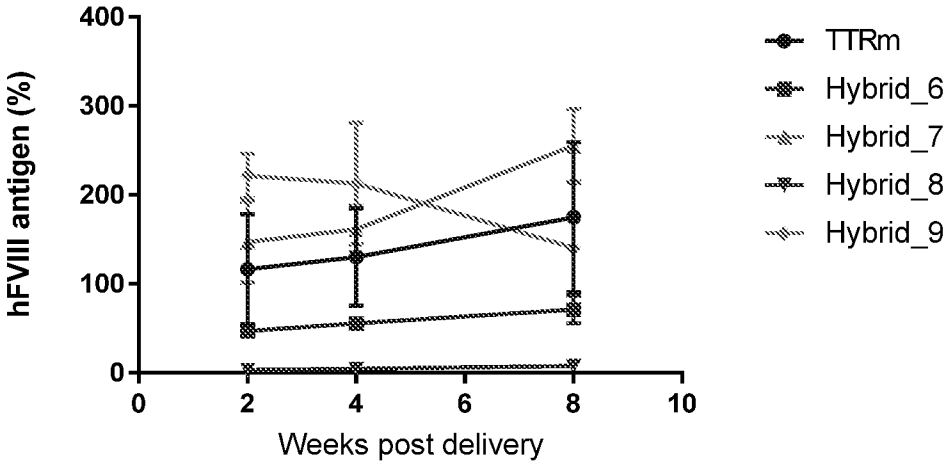


Figure 5

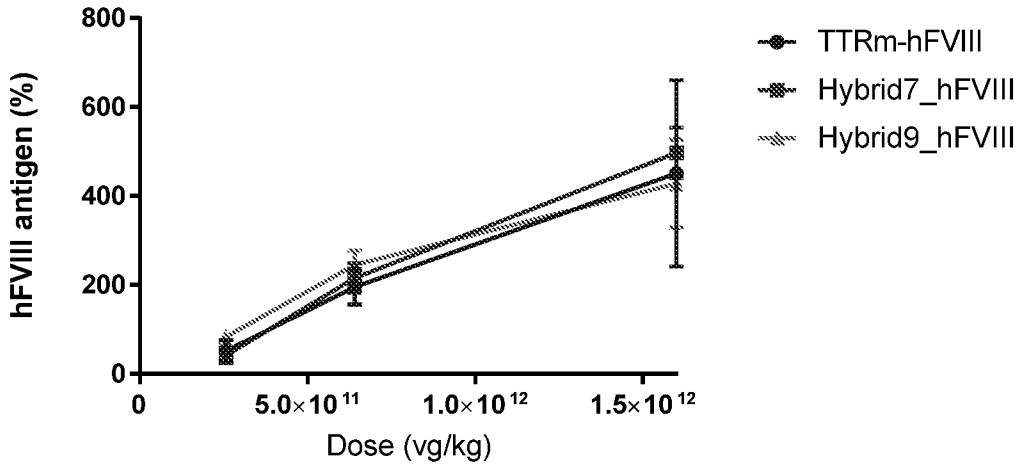


Figure 6

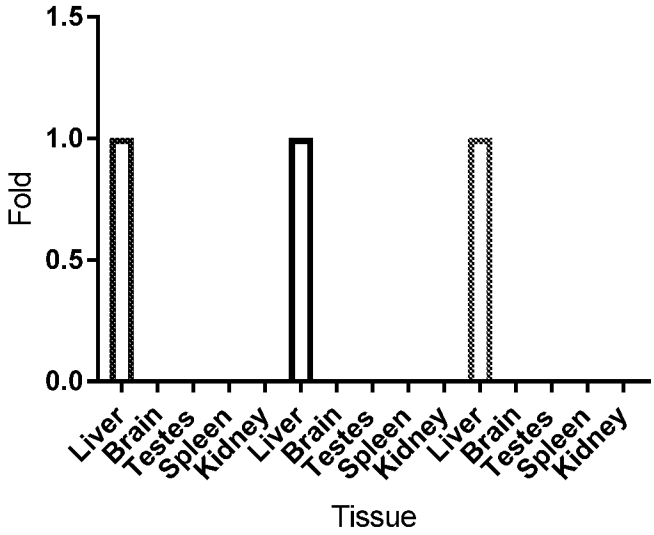


Figure 7

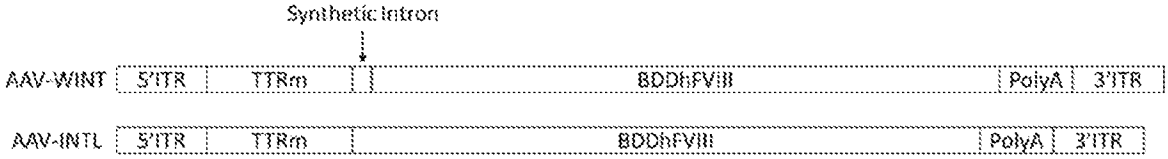


Figure 8

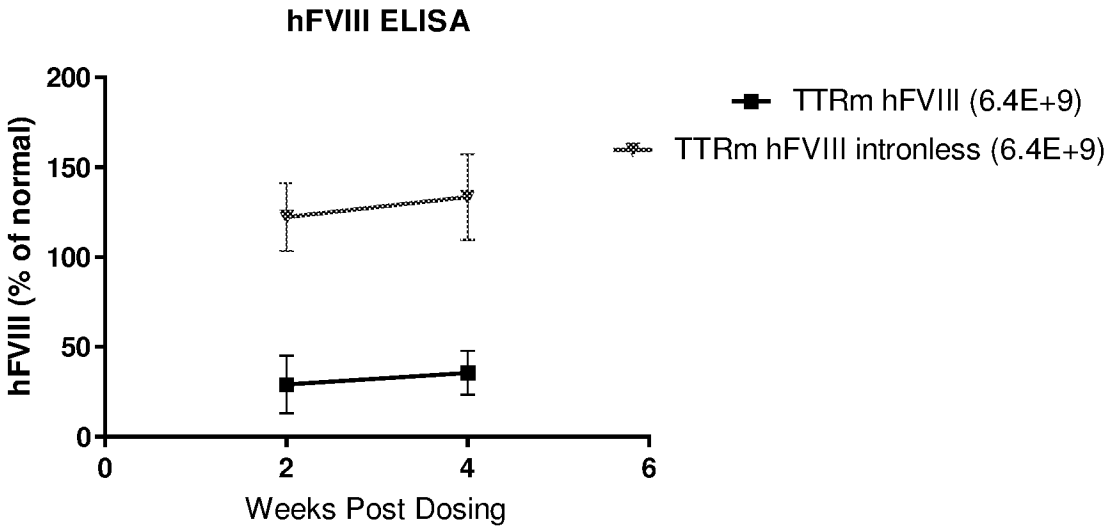


Figure 9

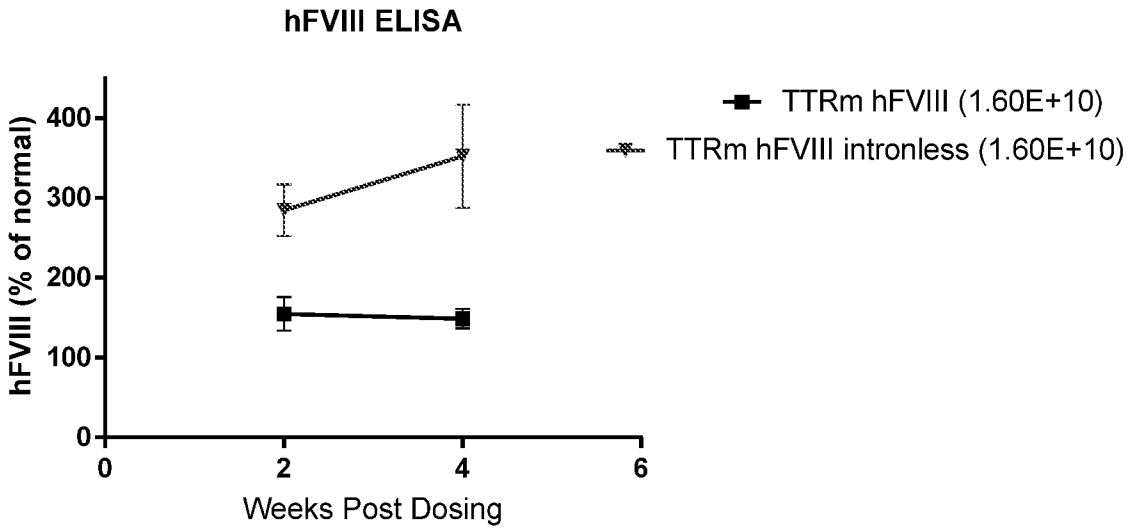


Figure 10

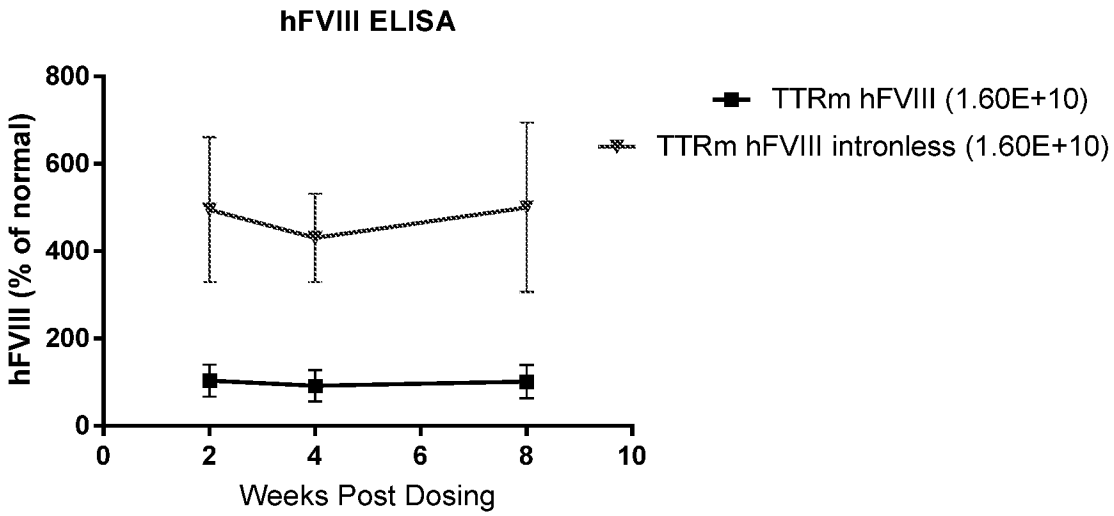


Figure 11

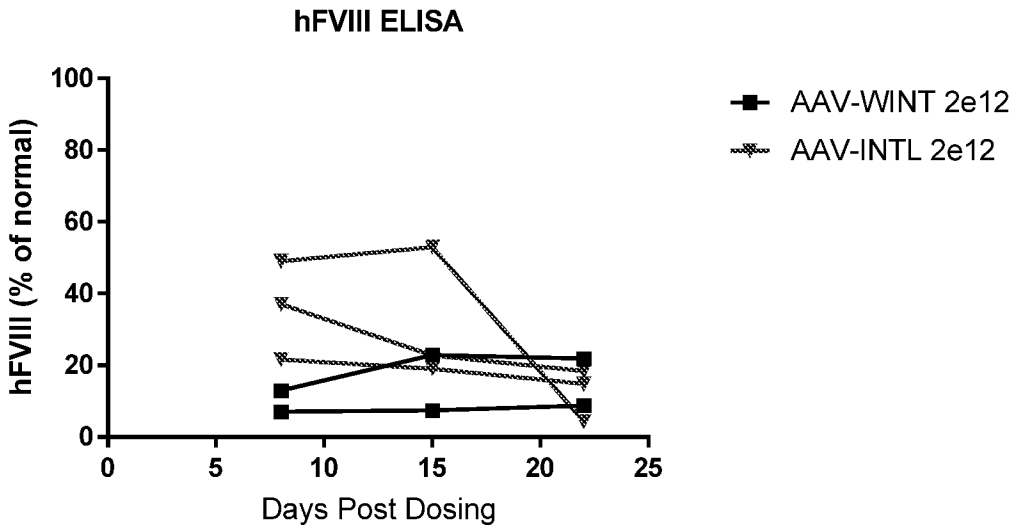


Figure 12

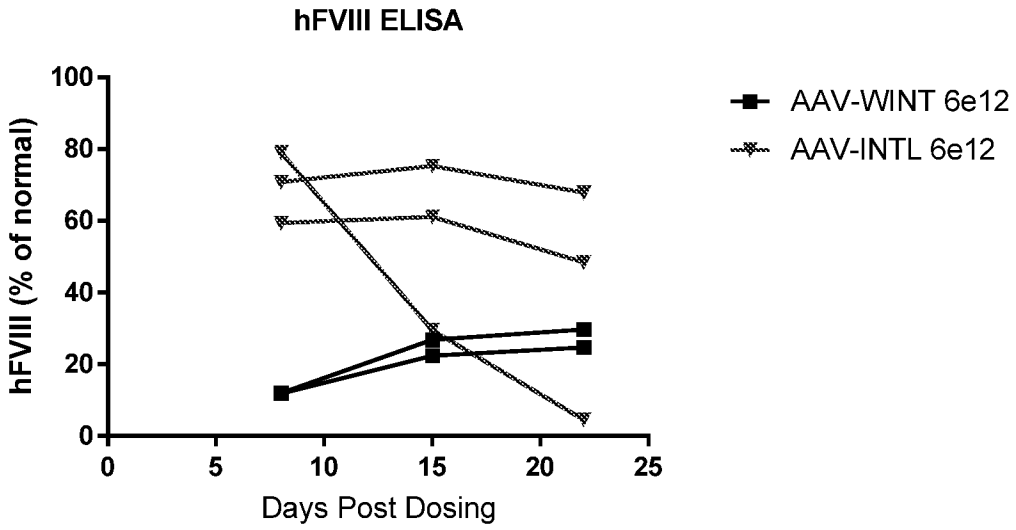


Figure 13

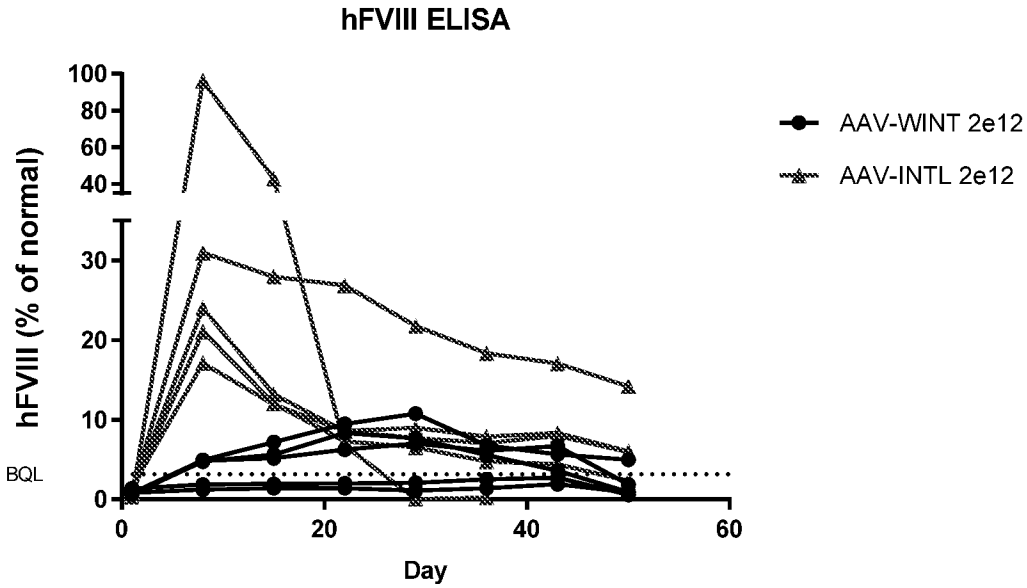


Figure 14

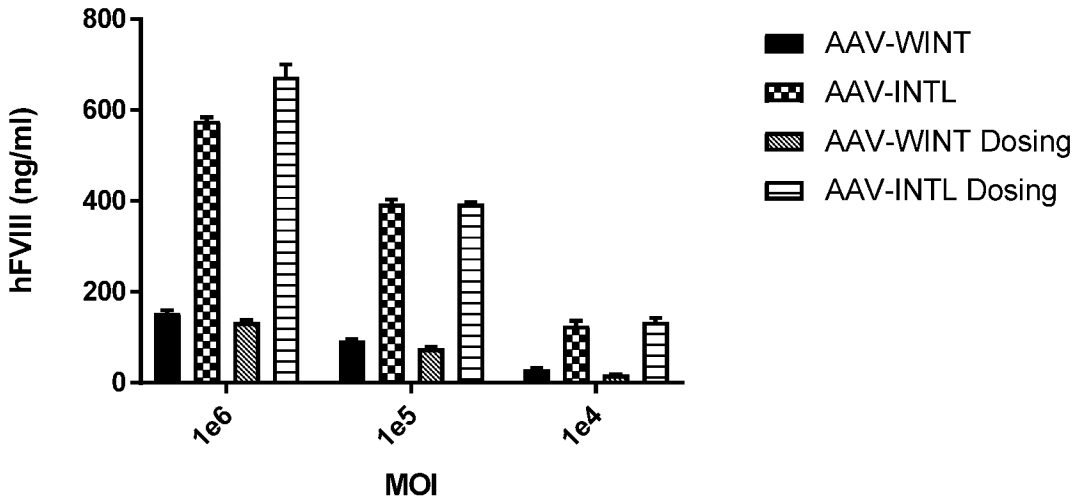


Figure 15

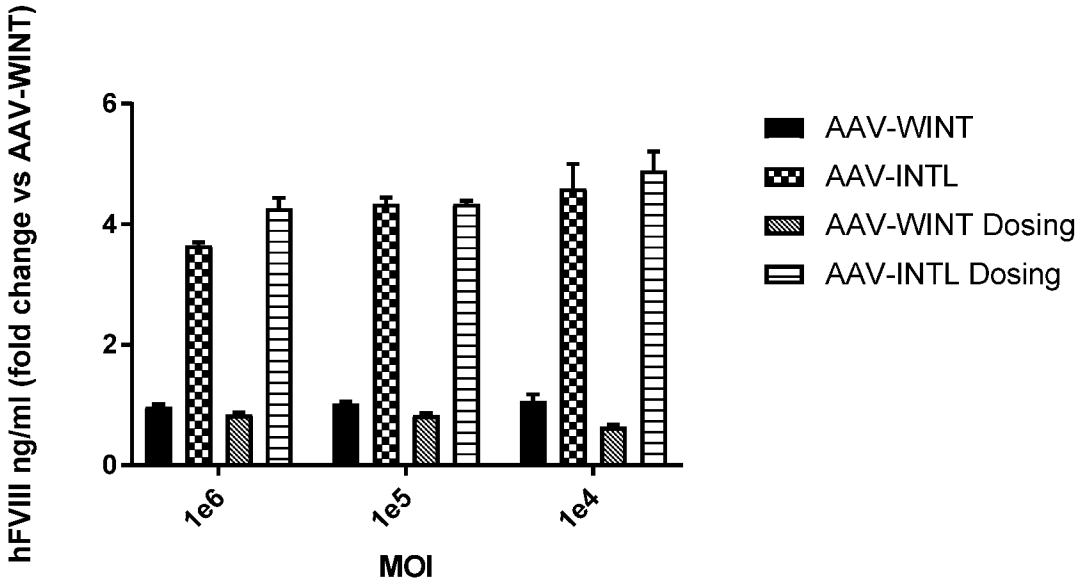


Figure 16

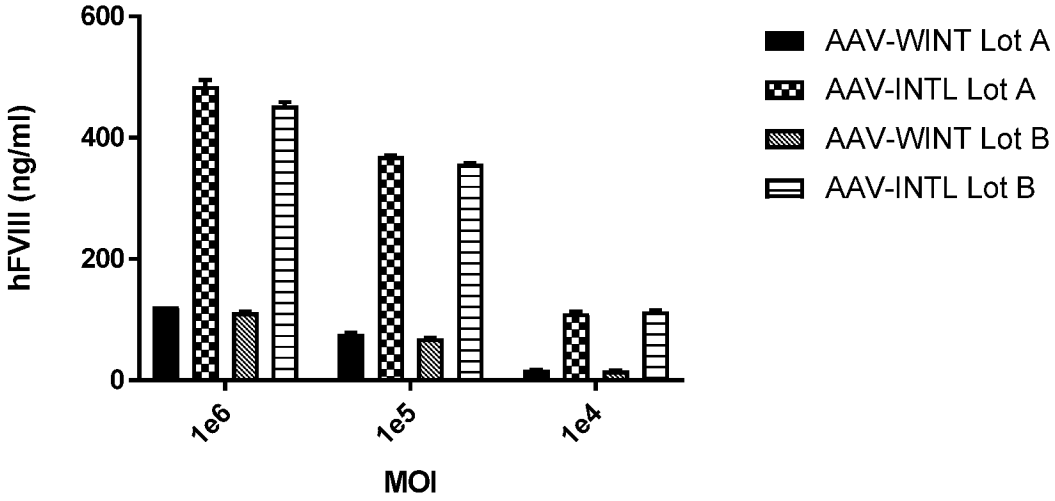


Figure 17

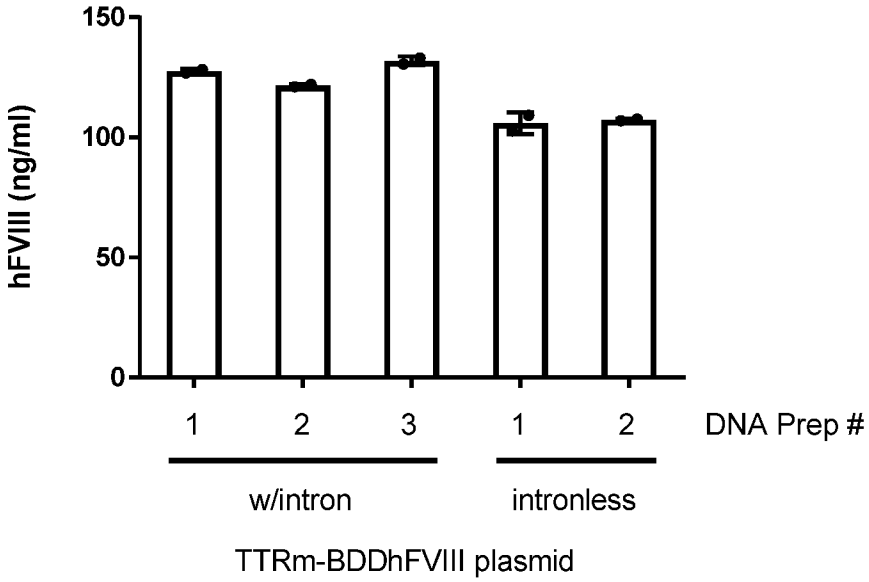
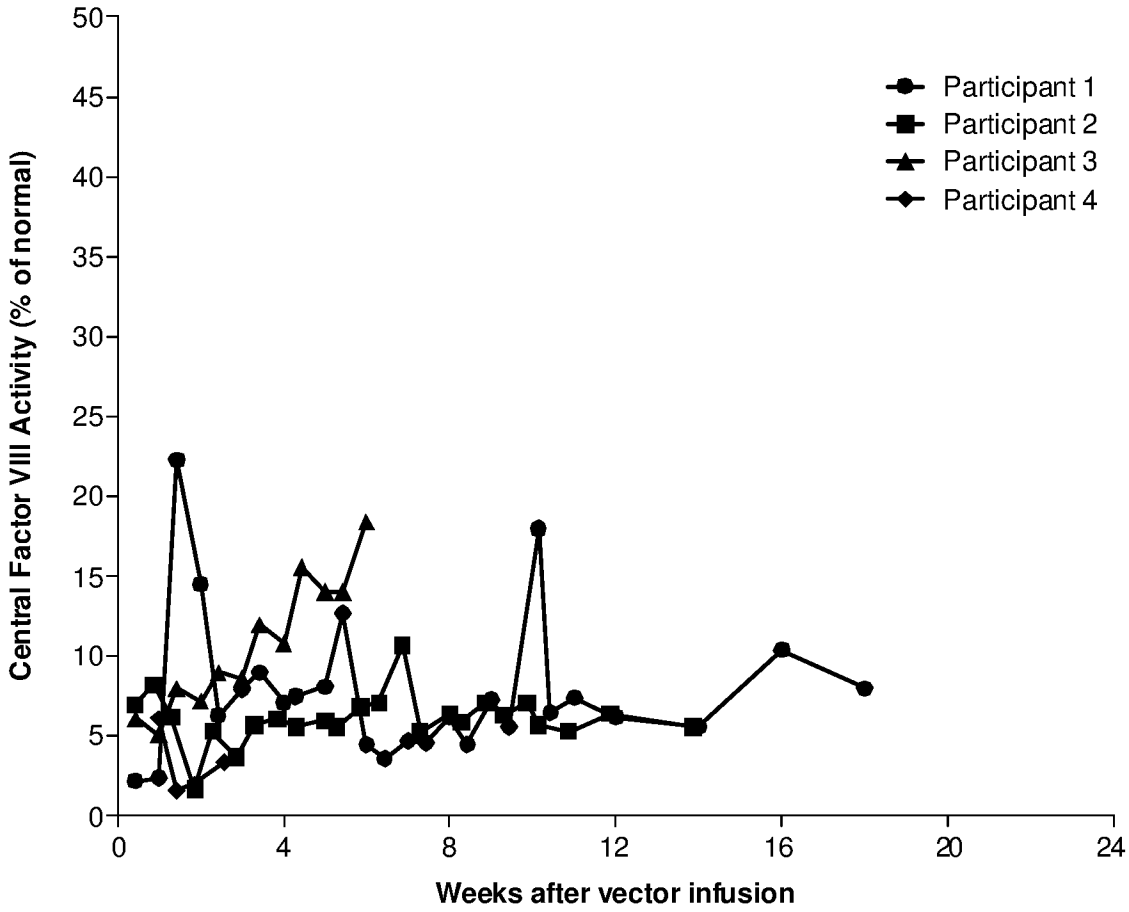

150
100
50
0
hFVIII (ng/ml)
1
2
3
1
2
DNA Prep #
w/intron
intronless
TTRm-BDDhFVIII plasmid

Figure 18


Central Factor VIII Activity (% of normal)
50
45
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25
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15
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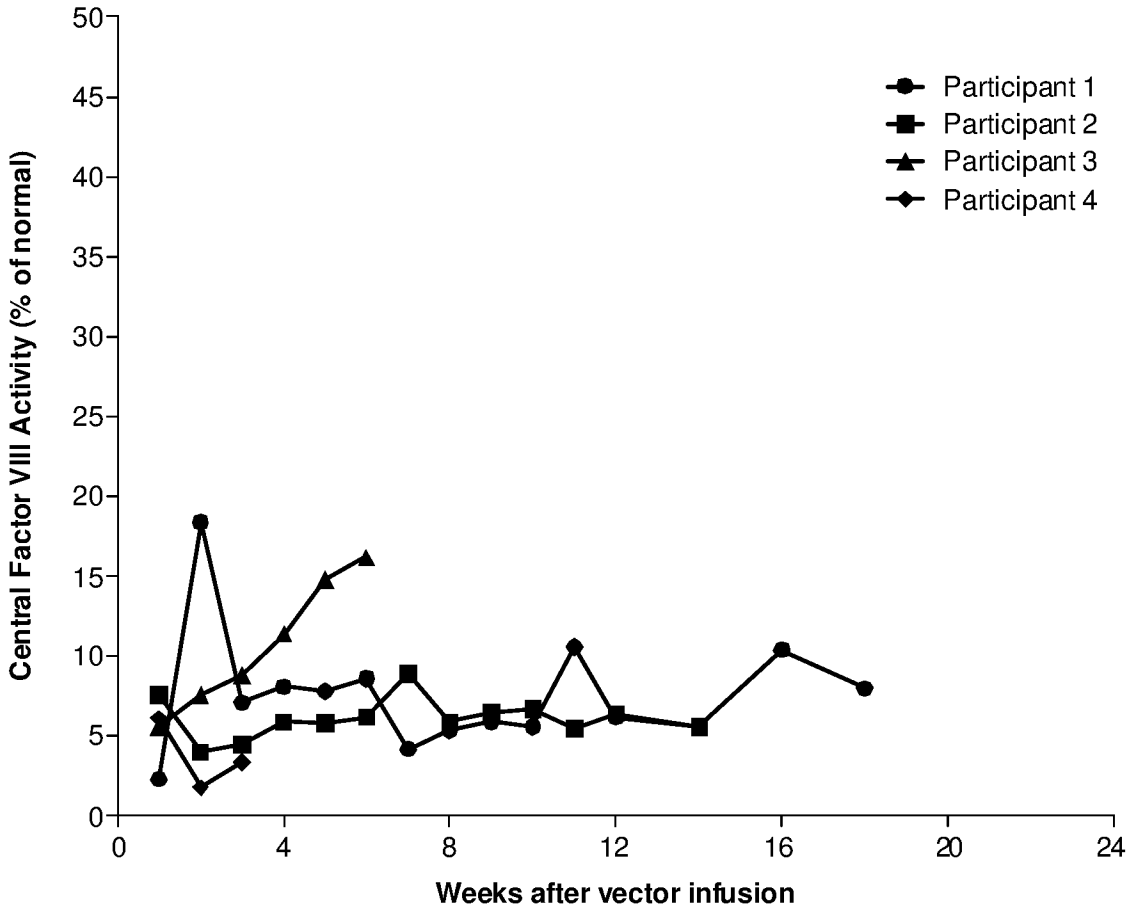
12
16
20
24
Weeks after vector infusion
Participant 1
Participant 2
Participant 3
Participant 4

Figure 19


Central Factor VIII Activity (% of normal)
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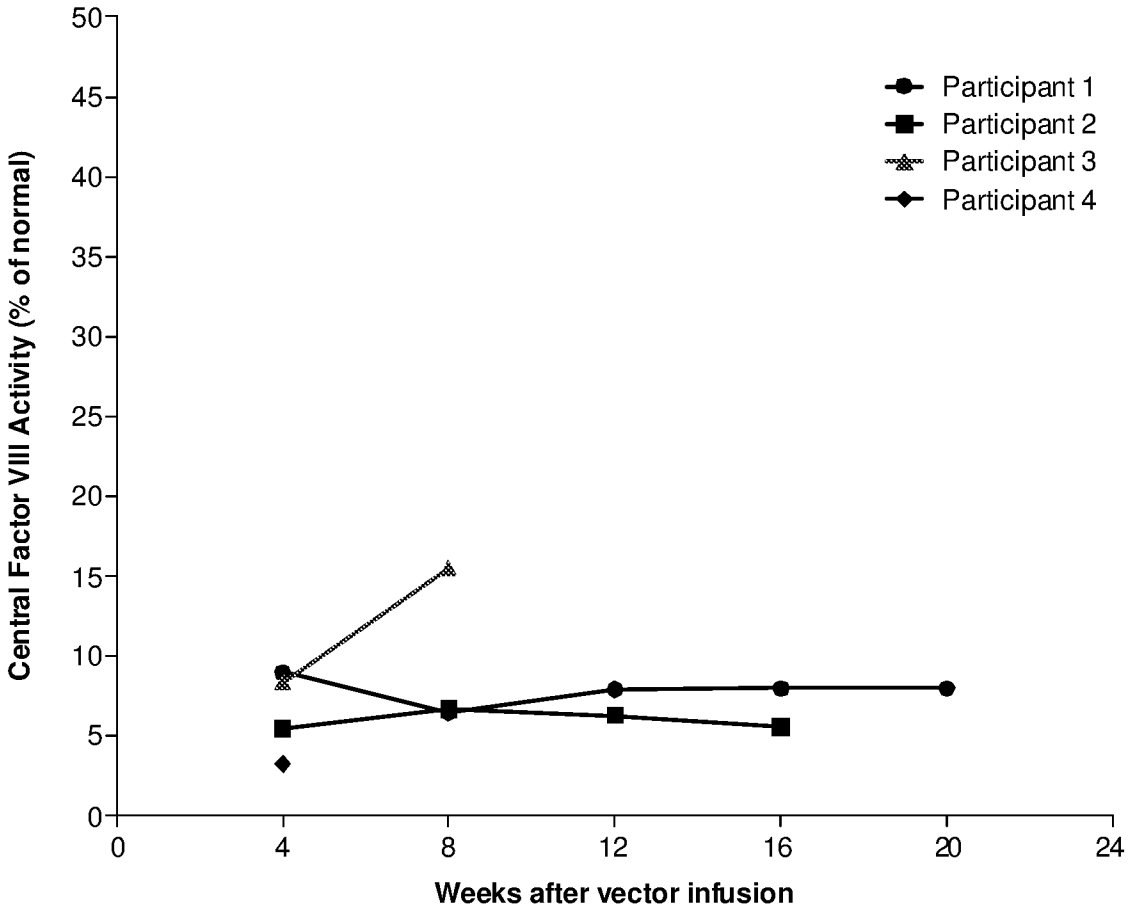
24
Weeks after vector infusion
Participant 1
Participant 2
Participant 3
Participant 4

Figure 20


Central Factor VIII Activity (% of normal)
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16
20
24
Weeks after vector infusion
Participant 1
Participant 2
Participant 3
Participant 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/48032

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 38/37, A61K 45/06, C12N 15/86, C12N 7/00, A61K 48/00 (2019.01)

CPC - A61K 48/0066, C07K 14/755, C12N 2710/10342, C12N 2710/10343, C12N 2750/14122, C12N 2750/14143, C12N 2750/14145

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2017/075619 A1 (SPARK THERAPEUTICS INC) 4 May 2017 (04.05.2017) Claim 1; Claim 37; SEQ ID NO:23	1-4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 January 2020	17 JAN 2020
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Lee Young Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/48032

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 9-14, 50, 54-104
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
----Please see continuation in first extra sheet ----------

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-4, limited to expression cassette comprising a sequence at least 98% identical to the sequence of SEQ ID NO: 1.

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US 19/48032

Continuation of Box No. III. Observations where unity of invention is lacking.

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I+, Claims 1-8, directed to an expression cassette comprising a nucleic acid sequence encoding a Factor VIII protein having a B domain deletion (FVIII-BDD). The expression cassette will be searched to the extent that the expression cassette comprises a sequence at least 98% identical to the sequence of SEQ ID NO: 1. It is believed that claims 1-4 encompass this first named invention (because this is the fist claimed embodiment for an expression cassette and contains an intron/5'UTR between the promoter and coding sequence, thus excluding claims 5-6 from the first embodiment), and thus these claims will be searched without fee to the extent that the expression cassette comprises a sequence at least 98% identical to the sequence of SEQ ID NO: 1, and further contains the promoter of SEQ ID NO:3, and the coding region of SEQ ID NO:77. Additional expression cassette(s) with alternate regulatory element(s) and/or alternate intron/5'UTR arrangements will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected expression cassette(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be an expression cassette comprising a regulatory element operably linked to a nucleic acid sequence encoding a Factor VIII protein having a B domain deletion (FVIIIBDD), wherein no intron is present between said regulatory element and said nucleic acid sequence, and wherein said expression cassette comprises a sequence at least 91 % identical to SEQ ID NO:1 (Claim 5). Another exemplary election would be an expression cassette comprising
a. a regulatory element at least 90% identical to the sequence of SEQ ID NO:2, and
b. a nucleic acid sequence encoding a Factor VIII protein having a B domain deletion (FVIII-BDD), said nucleic acid sequence having at least 90% identity to the sequence of SEQ ID NO:77, wherein said regulatory element is operably linked to said nucleic acid sequence, and wherein no intron is present between said regulatory element and said nucleic acid sequence (Claims 5-8).

Group II+, claims 15-49, 51-53, directed to a nucleic acid sequence modified to have fewer cytosine-guanine dinucleotides (CpGs). Group II+ will be searched upon payment of additional fees. The polynucleotide may be searched, for example, to encompass SEQ ID NO:2 with one fewer cytosine-guanine dinucleotides (CpGs) corresponding to the first CpG from the 5' end in SEQ ID NO:2, for an additional fee and election as such. It is believed that claims 15-16, 34(in part) read on this exemplary invention. Additional polynucleotides will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected polynucleotide(s). Failure to clearly identify how any paid additional invention fees are to be applied to the"+" group(s) will result in only the first claimed invention to be searched. Another exemplary election would be a polynucleotide comprising a nucleic acid sequence at least 95% identical to the sequence of SEQ ID NO:4 (claim 51).

The inventions listed as Group I+ and Group II+ do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special technical features

Group I+ has the special technical feature of an expression cassette, that is not required by Group II+.

Group II+ has the special technical feature of nucleic acid sequence modified to have fewer CpGs, that is not required by Group I+.

Additionally, the inventions of Group I+ and Group II+ include the special technical feature of a unique regulatory polynucleotide element with or without linkage to a nucleic acid sequence encoding a unique amino acid sequence, and is considered a distinct technical feature.

Shared technical features

Groups I+ and Group II+ were considered to share the technical features of including: a regulatory element polynucleotide sequence, these shared technical features are previously disclosed by prior art as discussed below.

No technical features are shared between the polynucleotide sequences of Groups I+ and II+ and, accordingly, these groups lack unity a priori.

Additionally, even if Group I+ was considered to share the technical features of including: an expression cassette comprising a nucleic acid sequence encoding a Factor VIII protein having a B domain deletion (FVIII-BDD) operably linked to a regulatory element; wherein no intron is present between said regulatory element and said nucleic acid sequence; and

additionally, even if Group II+ was considered to share the technical features of including: a regulatory polynucleotide modified to have fewer cytosine-guanine dinucleotides (CpGs), these shared technical features are previously disclosed by US 2017/0216408 A1 to SPARK THERAPEUTICS, INC. (hereinafter 'Spark'), in view of US 2004/0219677 A1 to Drocourt et al., (hereinafter 'Drocourt'), as discussed below.

--------please see continuation on next extra sheet-----

Continuation of Box No. III. Observations where unity of invention is lacking.

---continued from previous sheet----------

Spark teaches CpG reduced nucleic acid variants encoding FVIII protein having a B domain deletion and expression cassettes comprising such nucleic acid with or without an intron (Abstract - 'CpG reduced nucleic acid variants encoding FVIII protein and methods of use thereof are disclosed.'; Claim 107 - 'A nucleic acid variant encoding Factor VIII (FVIII) having a B domain deletion, wherein the nucleic acid variant has 92% or greater identity to SEQ ID NO:7.'; Claim 109 - 'The nucleic acid variant of claim 107, wherein the nucleic acid variant has 20 or fewer cytosine-guanine dinucleotides (CpGs).'; para [0008] - 'Such CpG reduced nucleic acid variants include variants that exhibit increased expression (e.g., 1-5 fold increased expression) compared to codon-optimized FVIII nucleic acids such as FVIII-CO3 (SEQ ID NO:21), when transferred into cells, leading to increased FVIII protein secretion and therefore increased activity.'; Claim 118 - 'The AAV vector of claim 117, wherein said AAV vector comprises:
a) an AAV capsid serotype; and/or
b) an intron; and/or
c) an expression control element operably linked to the nucleic acid variant; and/or
d) one or more adeno-associated virus (AAV) inverted terminal repeats (ITRs), wherein the AAV ITR(s) flanks the 5' or 3' terminus of the nucleic acid variant.'; Claim 122 - 'The AAV vector of claim 118, wherein the expression control element comprises a TTR promoter or mutant TTR promoter.'; para [0064] - 'An example of a recombinant polynucleotide would be where a CpG reduced nucleic acid encoding a FVIII protein is cloned into a vector, with or without 5', 3' and/or intron regions that the gene is normally associated within the viral (e.g., AAV) genome.'). Spark does not expressly teach a promoter with reduced CpG.
Drocourt teaches a promoter devoid of CpG (Abstract - 'The invention relates to a new series of bacterial plasmid vectors which are fully devoid of CpG'; para [0008] - 'The present application thus relates to methods for producing such plasmids, and also to the elements constituting these plasmids, namely genes devoid of CpG which can be expressed in E. coli, promoters devoid of CpG which are suitable for the expression of said genes, and origins of replication devoid of CpG which are suitable for the bacterial transformation of said plasmids.'). Since Spark teaches CpG reduced nucleic acid variants exhibit increased expression (para [0008] - 'Such CpG reduced nucleic acid variants include variants that exhibit increased expression (e.g., 1-5 fold increased expression) compared to codon-optimized FVIII nucleic acids such as FVIII-CO3 (SEQ ID NO:21), when transferred into cells, leading to increased FVIII protein secretion and therefore increased activity.'), it would have been obvious to one of ordinary skill in the art that CpG reduced nucleic acid variants could further include a CpG reduced promoter in a CpG-free plasmid according to Drocourt, to further aid increased expression of CpG reduced DNA.

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Group I+ and II+ inventions lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.

NOTE, continuation of number 4 above: claims 9-14, 50, 54-104 are held unsearchable because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).