(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 October 2016 (20.10.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/168728 A2

(51) International Patent Classification:
A61K 48/00 (2006.01) *C12N 9/64* (2006.01)
C07K 14/755 (2006.01)

(21) International Application Number:
PCT/US2016/027931

(22) International Filing Date:
15 April 2016 (15.04.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

62/148,696	16 April 2015 (16.04.2015)	US
62/202,133	6 August 2015 (06.08.2015)	US
62/212,634	1 September 2015 (01.09.2015)	US

(71) Applicants: **EMORY UNIVERSITY** [US/US]; 1599 Clifton Road Ne, 4th Floor, Atlanta, GA 30322 (US). **CHILDREN'S HEALTHCARE OF ATLANTA, INC.** [US/US]; 1600 Tullie Circle NE, Atlanta, GA 30329 (US).

(72) Inventors: **DOERING, Christopher, B.**; 1599 Clifton Road NE, 4th Floor, Atlanta, GA 30322 (US). **SPENCER, Trent, H.**; 1599 Clifton Road NE, 4th Floor, Atlanta, GA 30322 (US). **BROWN, Harrison, C.**; 1599 Clifton Road NE, 4th Floor, Atlanta, GA 30322 (US).

(74) Agent: **SCOTT, Gregory, K.**; Klarqueist Sparkman, LLP, One World Trade Center, Suite 1600, 121 Sw Salmon Street, Portland, OR 97204 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2016/168728 A2

(54) Title: RECOMBINANT PROMOTERS AND VECTORS FOR PROTEIN EXPRESSION IN LIVER AND USE THEREOF

(57) Abstract: Disclosed herein are recombinant viral vectors comprising a liver specific promotor in operable combination with a heterologous nucleic acid sequence encoding a protein, such as a clotting factor. Methods of treating a subject with a clotting disorder, such as hemophilia A or hemophilia B, are also provided.

RECOMBINANT PROMOTERS AND VECTORS FOR PROTEIN EXPRESSION IN LIVER AND USE THEREOF

RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 62/202,133, filed August 6, 2015, U.S. Provisional Application No. 62/212,634, filed September 1, 2015, and U.S. Provisional Application No. 62/148,696, filed April 16, 2015. Each of the provisional patent applications is incorporated by reference in its entirety.

FIELD OF THE DISCLOSURE

This relates to recombinant promoters and vectors transgene expression, as well as recombinant nucleic acid molecules encoding novel clotting factors.

ACKNOWLEDGMENT OF GOVERNMENT SUPPORT

This invention was made with government support under Grant Nos. R01 HL092179 U54 HL112309 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

Mutations in the clotting factor VIII (fVIII) gene result in a decreased or defective clotting factor (fVIII) protein that gives rise to hemophilia A, which is characterized by uncontrolled bleeding. Hemophilia B is similarly associated with clotting factor IX (fIX). Treatment of hemophilia A typically entails lifelong, multi-weekly intravenous infusion of either human plasma-derived or recombinant fVIII product. Due to the high cost, less than 30% of the global hemophilia A population receives this form of treatment. Furthermore, about 25% of patients treated with fVIII replacement products develop neutralizing antibodies that render future treatment ineffective. Thus, there is a need to identify improved therapies.

Gene therapies are typically based on genetically engineering viruses designed to deliver functional transgenes to the patient so that their own cells can biosynthesize missing or defective proteins. Clinical advancements have been made using recombinant adeno-associated viral (rAAV) vectors for the expression of fIX in the liver; however, using rAAV for fVIII expression for patients with hemophilia A has been challenging due to inefficient biosynthesis of human fVIII (hfVIII). Recombinant adeno-associated viral (rAAV) vectors produce capsids that have a limited space for encapsulating nucleic acids. FVIII is a large glycoprotein, and the rAAV sequences needed for encoding and expressing fVIII typically exceed capsid packing capacity.

SUMMARY

Disclosed herein are embodiments of a novel recombinant nucleic acid molecule comprising a promoter that has been optimized to be of minimal length and to promote tissue-specific protein expression. In several embodiments, the promoter can be a liver-specific promoter that promotes substantially more protein expression in liver and liver cells than in other tissue types. In some embodiments, promoter can be included in a viral vector (such as an adeno-associated virus vector) in operable combination with a heterologous nucleic acid sequence encoding a protein of interest in order to promote expression of the protein of interest, for example in liver tissue and/or cells.

In some embodiments, the recombinant nucleic acid molecule can comprise a promoter comprising a first response element that comprises a set of transcription factor (TF) binding sites including: a HNF1a TF binding site, a HNF1-1 TF binding site, a HNF4 TF binding site, a HNF3a TF binding site, a HNF1-2 TF binding site, a HNF3-2 TF binding site, a HP1 TF binding site, a TATA box; and a Transcription Start Site. In some embodiments, the HNF1a TF binding site comprises or consists of nucleotides 1-12 of SEQ ID NO: 4; the HNF1-1 TF binding site comprises or consists of nucleotides 16-23 of SEQ ID NO: 4; the HNF4 TF binding site comprises or consists of nucleotides 26-36 of SEQ ID NO: 4; the HNF3a TF binding site comprises or consists of nucleotides 39-45 of SEQ ID NO: 4; the HNF1-2 TF binding site comprises or consists of nucleotides 48-62 of SEQ ID NO: 4; the HNF3-2 TF binding site comprises or consists of nucleotides 65-71 of SEQ ID NO: 4; the HP1 TF binding site comprises or consists of nucleotides 75-87 of SEQ ID NO: 4; the TATA box comprises or consists of nucleotides 108-114 of SEQ ID NO: 4; and/or the Transcription Start Site (TSS) comprises or consists of nucleotides 116-146 of SEQ ID NO: 4. In some embodiments, the first response element can be of no more than 160 nucleotides in length (such as no more than 150 nucleotides in length, such as 146 nucleotides in length).

In some embodiments, the first response element comprises, from 5' to 3', the HNF1a TF binding site, the HNF1-1 TF binding site, the HNF4 TF binding site, the HNF3a TF binding site, the HNF1-2 TF binding site, the HNF3-2 TF binding site, the HP1 TF binding site, the TATA box, and the Transcription Start Site (TSS).

In some embodiments, the recombinant nucleic acid molecule comprises a promoter comprising or consisting of the nucleic acid sequence set forth as SEQ ID NO: 4 (HCB), or a sequence at least 90% identical thereto.

In some embodiments, the recombinant nucleic acid molecule can comprise a promoter comprising the first response element as discussed above, and can further comprise a second response element. The second response element can comprise, for example, a HSh response element (for example, comprising or consisting of the nucleotide sequence set forth as SEQ ID NO: 111, or a sequence at least 90% identical thereto), a 5'HS response element (for example, comprising or consisting of the nucleotide sequence set forth as nucleotides 6-32 of SEQ ID NO: 111, or a sequence at least 90% identical thereto), or a 3'HS response element (for example, comprising or consisting of the nucleotide sequence set forth as nucleotides 44-68 of SEQ ID NO: 111, or a sequence at least 90% identical thereto).

In some embodiments, the recombinant nucleic acid molecule comprises a promoter comprising or consisting of the nucleic acid sequence set forth as one of SEQ ID NO: 102 (HSh-HCB), SEQ ID NO: 104 (5'HSh-HCB), or SEQ ID NO: 103 (3'HSh-HCB), or a sequence at least 90% identical to one of SEQ ID NO: 102 (HSh-HCB), SEQ ID NO: 104 (5'HSh-HCB), or SEQ ID NO: 103 (3'HSh-HCB). In additional embodiments, the recombinant nucleic acid molecule comprises a promoter comprising or consisting of the nucleic acid sequence set forth as one of SEQ ID NO: 5 (shortABP-HP1-God-TSS), SEQ ID NO: 7 (ABP-HP1-God-TSS), SEQ ID NO: 105 (HSh-SynO-TSS), SEQ ID NO: 106 (sHS-SynO-TSS), SEQ ID NO: 107 (Agro), SEQ ID NO: 108 (HS-SynO-TSS), or SEQ ID NO: 112 (HNF1-ShortABPExact-SynO-TSS-Int), or a sequence at least 90% identical to one of SEQ ID NO: 5 (shortABP-HP1-God-TSS), SEQ ID NO: 7 (ABP-HP1-God-TSS), SEQ ID NO: 105 (HSh-SynO-TSS), SEQ ID NO: 106 (sHS-SynO-TSS), SEQ ID NO: 107 (Agro), SEQ ID NO: 108 (HS-SynO-TSS), or SEQ ID NO: 112 (HNF1-ShortABPExact-SynO-TSS-Int).

In some embodiments, promoter can be included in a vector, such as a viral vector (for example, an adeno-associated virus vector). In some embodiments, the promoter is included on the vector in operable combination with a heterologous nucleic acid sequence encoding a protein of interest in order to promote expression of the protein of interest, for example in liver tissue and/or cells. In some embodiments, the protein of interest can be a clotting factor, such as fVIII or fIX or variant thereof, such as a fVIII variant comprising fVIII A1, A2, A3, C1, and C2 domains, with the A2 and A3 domains joined by a peptide linker, and deletion of the fVIII B domain. In some embodiments, the protein of interest can be a fVIII variant and the heterologous nucleic molecule can comprise or consist of the nucleic acid sequence set forth as SEQ ID NO: 2, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 125, or SEQ ID NO: 126, or a nucleic acid sequence at least 90% identical to SEQ ID NO: 2, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 125, or SEQ ID NO: 126. In some embodiments, the protein of interest can be a fIX and the heterologous nucleic molecule can comprise or consist of the nucleic acid sequence set forth as SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 124, or SEQ ID NO: 127, or a nucleic acid sequence at least 90% identical SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 124, or SEQ ID NO: 127.

In some embodiments, the vector can be a recombinant AAV vector comprising a genome comprising a nucleic acid molecule encoding any of the liver-specific promoters provided herein (such as the HCB promoter, SEQ ID NO: 4) operably linked to a heterologous nucleic molecule encoding a fVIII variant, wherein the heterologous nucleic acid molecule comprises or consists of the nucleic acid sequence set forth as SEQ ID NO: 2, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 125, or SEQ ID NO: 126, or a nucleic acid sequence at least 90% identical to SEQ ID NO: 2, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 125, or SEQ ID NO: 126. Is several such embodiments, the recombinant AAV genome (from 5' to 3' ITR) is no more than 5.1, 5.0, 4.9, 4.8, 4.7, 4.6, or 4.5 kb in length.

In some embodiments, the vector can be a recombinant AAV vector comprising a genome comprising a nucleic acid molecule encoding any of the liver-specific promoters provided herein (such as the HCB promoter, SEQ ID NO: 4) operably linked to a heterologous nucleic molecule encoding a fIX variant, wherein the heterologous nucleic acid molecule comprises or consists of the nucleic acid

sequence set forth as SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 124, or SEQ ID NO: 127, or a nucleic acid sequence at least 90% identical SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 124, or SEQ ID NO: 127. In several such embodiments, the recombinant AAV genome (from 5' to 3' ITR) is no more than 5.1, 5.0, 4.9, 4.8, 4.7, 4.6, or 4.5 kb in length.

In some embodiments, a method of inducing blood clotting in a subject in need thereof is provided. The method comprises administering to the subject a therapeutically effective amount of a vector (such as an AAV vector) encoding a clotting factor as described herein. In some embodiments, the subject is a subject with a clotting disorder, such as hemophilia A or hemophilia B. In some embodiments, the clotting disorder is hemophilia A and the subject is administered a vector comprising a nucleic acid molecule encoding a protein with fVIII activity. In other embodiments, the clotting disorder is hemophilia B and the subject is administered a vector comprising a nucleic acid molecule encoding a protein with fIX activity.

The foregoing and other features and advantages of this disclosure will become more apparent from the following detailed description of several embodiments which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE FIGURES

FIGs. 1A and 1B illustrate a sequence alignment of the A1 and A3 domains for human and porcine orthologues of the ET3 variant fVIII protein. (FIG. 1A) Sequence alignment of the A1 domain for human (upper sequence, SEQ ID NO: 13) and porcine (middle sequence, SEQ ID NO: 14) ET3 variant of fVIII. The lower sequence shows identical residues. Amino acid sequence alignments for the signal peptide (N-terminal bar), heavy chain acidic domain (C-terminal bar), human (top) and ET3 (bottom) fVIII are shown. Disulfide linkages are noted by the lines connecting cysteine residues. Places where either human, ET3 or both sequences encode an N-linked glycosylation attachment site (N-X-S/T) are outlined with a box. (FIG. 1B) Sequence alignment of the A3 domain for human (upper sequence, SEQ ID NO: 15) and porcine (middle sequence, SEQ ID NO: 16) ET3 variant of fVIII. The lower sequence shows identical residues. Amino acid sequence alignments for the activation peptide (bar), human (top) and ET3 (bottom) fVIII are shown. Disulfide linkages are noted by the lines connecting cysteine residues. Places where either human, ET3 or both sequences encode an N-linked glycosylation attachment site (N-X-S/T) are outlined with a box.

FIGs. 2A-2C show liver, total human, and myeloid specific codon bias tables.

FIG. 3A shows *in vitro* expression data in HepG2 cells indicating that liver specific codon optimization improves expression for the HSQ and ET3 variants of the fVIII protein.

FIG. 3B shows *in vivo* data for codon optimized HSQ and ET3 indicating increased expression of fVIII following hydrodynamic injection of an AAV-vector encoding liver codon optimized variants of the HSQ and ET3 fVIII protein into mice.

FIG. 4 illustrates AAV vectors encoding fVIII variants.

FIG. 5 shows *in vitro* expression data in HepG2 cells indicating that liver specific codon optimization, but not myeloid specific codon optimization, improves expression of HSQ and ET3 variants of the fVIII protein in liver cells.

FIG. 6 shows *in vitro* expression data in HepG2 cells indicating that liver specific codon optimization, but not myeloid specific codon optimization, improves expression of HSQ and ET3 variants of the fVIII protein in liver cells.

FIG. 7 shows *in vitro* expression data in HepG2 cells indicating that liver specific codon optimization, but not human specific codon optimization, improves expression of fIX protein in liver cells.

FIG. 8 illustrates promotors comprising an ABP enhancer or a shortened ABP enhancer.

FIGs. 9A and 9B show data concerning use of AAV vectors containing the indicated promoters for expression of fVIII. (FIG. 9A) *In vitro* expression data in HepG2 cells. (FIG. 9B) *In vivo* expression of fVIII following hydrodynamic injection of an AAV-vector.

FIG. 10 illustrates response element sequences and components of the indicated response elements.

FIGs. 11A and 11B illustrate promotor sequences and components of the indicated promoters.

FIGs. 12A and 12B show data concerning use of AAV vectors containing the indicated promoters for expression of fVIII. (FIG. 12A) *In vitro* expression data in HepG2 cells. (FIG. 12B) *In vivo* expression of fVIII following hydrodynamic injection of an AAV-vector.

FIG. 13 illustrates promotor sequences and components of the indicated promoters.

FIGs. 14A and 14B show data concerning use of AAV vectors containing the indicated promoters for expression of fVIII. (FIG. 14A) *In vitro* expression data in HepG2 cells. (FIG. 14B) *In vivo* expression of fVIII following hydrodynamic injection of an AAV-vector.

FIGs. 15 and 16 show schematic diagrams illustrating the structure of liver-specific enhancers (FIG. 15) and promoters including the liver specific enhancers (FIG. 16).

FIG. 17 shows data from *in vitro* assays concerning use of AAV vectors containing the indicated promoters for expression of fVIII in HepG2 cells.

FIG. 18 illustrates an AAV vectors encoding an fVIII variant.

FIG. 19 is a graph showing that transduction of mice with the AAV2-HCB-ET3-LCO-NCG-SpA vector (SEQ ID NO: 130) encoding liver codon-optimized ET3 with deleted CpG motifs leads to a significant increase in fVIII activity in transduced mice.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. The Sequence Listing is submitted as an ASCII text file in the form of the file named "Sequence.txt" (~148 kb), which was created on April 14, 2016 which is incorporated by reference herein.

DETAILED DESCRIPTION

There is a need to develop a safe and efficient gene transfer strategy for the treatment of hemophilia, such as hemophilia A and B, and acquired hemophilia. In the context of gene therapies for the treatment of hemophilia A, several obstacles have hindered the development of using an adeno-associated viral vector as the gene delivery vehicle, such as the limited DNA packaging capacity of the adeno-associated virus for the large fVIII transgene, and inefficient biosynthesis of human fVIII. Reported herein is an AAV-based transgene delivery system that utilizes improvements for the expression of fVIII in the context of liver-directed AAV gene transfer. These include: 1) The use a nucleotide coding sequence that has an improved codon usage bias for the human liver cell compared to the naturally occurring nucleotide sequence of fVIII; 2) Optimization of the codon usage to remove 5'-CG-3' dinucleotides and other deleterious cis-acting DNA motifs, *e.g.*, cryptic splice sites, TATA boxes, terminal signal, mRNA secondary structure, premature polyA signals, RNA instability motifs, internal ribosomal binding sites; and 3) Minimally sized, liver-directed promoters in order to reduce the size of the transgene so it may be used in the size-constrained environment of the adeno-associated viral vector system. The improvements may be generalized for the improved expression of any AAV transgene. In some embodiments, the AAV vector delivers efficacious expression of fVIII at viral doses not predicted to cause toxicity in humans.

In some embodiments, these improvements may be applied to fIX as well, especially for self-complimentary fIX vector designs. Self-complimentary designs have half the packaging capacity is single stranded designs, so vector size limitations (~2.4kb) become a concern even for fIX.

Prior work suggested that treatment of fVIII-deficient (hemophilia A) mice with an AAV vector encoding a modified form of fVIII (B-domain deleted) termed ET3 at vector doses ranging from $5x10^{11}$-$2x10^{13}$ vp/kg could theoretically correct their fVIII deficiency and bleeding phenotype (see Brown et al., "Bioengineered Factor FVIII Enables Long-Term Correction of Murine Hemophilia A Following Liver-Directed Adeno-Associated Viral Vector Delivery," *Molecular Therapy – Methods and Clinical Development.* 1:14036, 2014). However, due the oversized genome of ET3, the vector suffered from low titer manufacture and substantial inter-particle heterogeneity. The large size of the codon optimized ET3-AAV genome remained incompatible with efficient viral vector packaging. For AAV vectors, an AAV genome size of no more than 4.7-5.0 kb is preferred for higher yield and consistency than genomes exceeding 5.0kb. The B-domain deleted ET3 coding sequence is 4.4kb. However, with the addition of necessary viral and regulatory control elements, fVIII ET3-AAV genomes substantially exceeded the packaging capacity.

Disclosed herein for the first time is an fVIII (ET3 or other B-domain deleted variant)-AAV genome of less than 5.0kb in length that was developed to allow for both enhanced fVIII (or variant thereof) expression and efficient viral packaging. Multiple steps were taken to reduce the size of the AAV genome to acceptable levels. For example, a combinatorial transcription factor binding site assembly approach was used to create a panel of liver-specific promoters ranging in size. These promoters represent a 30-90% size reduction over currently utilized liver specific promoters such as HLP and HCR-

hAAT, which range in size from 250 to over 700 bases. Some of these promoters drive comparable or better transgene expression levels and specificity to that observed with HLP and HCR-hAAT.

A significant barrier to the development of successful clinical gene transfer-based therapeutics is the availability of naturally occurring or synthetic genetic elements capable of functional, and often cell type-directed or restricted, expression in the context of a vector-delivered nucleic acid cassette (see, e.g., Papadakis et al., "Promoters and control elements: designing expression cassettes for gene therapy," Curr Gene Ther., 4(1):89-113, 2004). It is generally believed that naturally existing promoters have been honed by evolution to drive finely tuned expression through the combination of multiple cis-regulatory sequences. In most living organisms, and especially eukaryotes with large genome sizes, there does not appear to be a driving force to limit promoter size and thus most endogenous promotes are spread over hundreds, and more often thousands, of DNA basepairs (bp). Due to their size, these endogenous, native gene promoters typically are not amenable to inclusion into gene therapy products due to size constraints.

Endogenous viral promoters on the other hand have evolved to possess an efficiency of strength and size than make them attractive for use in gene transfer technologies. Prominent examples include the cytomegalovirus (CMV) immediate early (IE) promoter, the adenovirus (Ad) major late promoter, simian virus 40 (SV40) promoter and Moloney murine leukemia virus (MoMLV) long terminal repeat (LTR). Each of these promoters can drive high-level transcription of exogenous heterologous transgenes in a variety of eukaryotic cell types. However, not surprisingly, eukaryotic cells have developed cellular defense mechanisms to effectively detect and inactivate (i.e. silence) viral promoters and thus these promoters perform more effectively in cell culture model systems than in vivo gene therapy applications.

For these reasons, there has been significant interest in the development of synthetic promoters, either generic (see, e.g., Juven-Gershon et al., "Rational design of a super core promoter that enhances gene expression," Nat Methods, 3(11):917-22, 2006; Schlabach et al., "Synthetic design of strong promoters." *PNAS*, 2010;107(6):2538-43, 2010), or tailored to specific gene therapy applications including hemophilia A and B (see, e.g., McIntosh et al., "Therapeutic levels of FVIII following a single peripheral vein administration of rAAV vector encoding a novel human factor VIII variant," Blood, 121(17):3335-44, 2013; Nair et al., "Computationally designed liver-specific transcriptional modules and hyperactive factor IX improve hepatic gene therapy," Blood, 123(20):3195-9, 2014).

Knowledge of promoters and enhances remains limited and currently it is not possible to computationally design an optimal promoter with any confidence. Studies such as those described by Juven-Gershon and Kadonaga have made progress defining optimized core promoter designs such as their Super Core Promoter 1 (SCP1) and are informative to the field. However, as we show in promoters described in the examples herein, which do not contain strong similarity to SCP1 in the core promoter domain, these sequences are not necessary for strong promoter function, at least in the context of liver-directed gene therapy applications (see also, Juven-Gershon et al., "Rational design of a super core promoter that enhances gene expression," Nat Methods, 3(11):917-22, 2006).

Most generic promoter development has focused on achieving an optimal balance of transcriptional potency with minimal size. In the field of liver-directed promoter design, the use of rational design approaches by McIntosh et al. and Nair et al. (*supra*) led to identification of promoters for

use in AAV-fVIII and AAV-fIX gene therapy approaches. However despite extensive study, both groups describe promoter designs that are significantly larger (≥ 252 bp) and no more (or less) potent than those described herein such as SEQ ID NO: 4 (HCB). Indeed, given the prior attempts to optimize promoter design, it was particularly surprising to identify promoters such as those described herein that are smaller that prior art promoters (such as the HLP promoter), yet equivalent or enhanced potency for driving transcription, particularly in the context of *in vivo* gene therapy applications. .

I. Terms

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

In order to facilitate review of the various embodiments of the disclosure, the following explanations of specific terms are provided:

5' and/or 3': Nucleic acid molecules (such as, DNA and RNA) are said to have "**5' ends**" and "**3' ends**" because mononucleotides are reacted to make polynucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester linkage. Therefore, one end of a linear polynucleotide is referred to as the "5' end" when its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring. The other end of a polynucleotide is referred to as the "3' end" when its 3' oxygen is not linked to a 5' phosphate of another mononucleotide pentose ring. Notwithstanding that a 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor, an internal nucleic acid sequence also may be said to have 5' and 3' ends.

In either a linear or circular nucleic acid molecule, discrete internal elements are referred to as being "**upstream**" or 5' of the "**downstream**" or 3' elements. With regard to DNA, this terminology reflects that transcription proceeds in a 5' to 3' direction along a DNA strand. Promoter and enhancer elements, which direct transcription of a linked gene, are generally located 5' or upstream of the coding region. However, enhancer elements can exert their effect even when located 3' of the promoter element and the coding region. Transcription termination and polyadenylation signals are located 3' or downstream of the coding region.

Adeno-associated virus (AAV): A small, replication-defective, non-enveloped virus that infects humans and some other primate species. AAV is not known to cause disease and elicits a very mild immune response. Gene therapy vectors that utilize AAV can infect both dividing and quiescent cells and can persist in an extrachromosomal state without integrating into the genome of the host cell. These features make AAV an attractive viral vector for gene therapy. There are currently 11 recognized serotypes of AAV (AAV1-11).

Administration/Administer: To provide or give a subject an agent, such as a therapeutic agent (*e.g.* a recombinant AAV), by any effective route. Exemplary routes of administration include, but are not limited to, injection (such as subcutaneous, intramuscular, intradermal, intraperitoneal, and intravenous), oral, intraductal, sublingual, rectal, transdermal, intranasal, vaginal and inhalation routes.

Bleeding Time Assay: An assay used to measure the amount of time it takes for a subject's blood to clot. A blood pressure cuff is placed on the upper arm and inflated. Two incisions are made on the lower arm. These are about 10 mm (less than 1/2 inch) long and 1 mm deep (just deep enough to cause minimal bleeding). The blood pressure cuff is immediately deflated. Blotting paper is touched to the cuts every 30 seconds until the bleeding stops. The length of time it takes for the cuts to stop bleeding is recorded. In normal, non-hemophiliacs, bleeding stops within about one to ten minutes and may vary from lab to lab, depending on how the assay is measured. In contrast, severe hemophiliacs having less than 1% of normal levels of the appropriate clotting factor have a whole blood clotting time of greater than 60 minutes. In mice, the bleeding time is assayed by transecting the tip of the tail and periodically touching a blotting paper until a clot is formed at the tip of the tail. Normal bleeding time is between 2-4 minutes. In contrast, hemophiliac mice having less than 1% of normal levels of the appropriate clotting factor have a bleeding time of greater than 15 minutes.

cDNA (complementary DNA): A piece of DNA lacking internal, non-coding segments (introns) and regulatory sequences that determine transcription. cDNA is synthesized in the laboratory by reverse transcription from messenger RNA extracted from cells. cDNA can also contain untranslated regions (UTRs) that are responsible for translational control in the corresponding RNA molecule.

Clotting disorder: A general term for a wide range of medical problems that lead to poor blood clotting and continuous bleeding. Doctors also refer to clotting disorders by terms such as, for example, coagulopathy, abnormal bleeding and bleeding disorders. Clotting disorders include any congenital, acquired or induced defect that results in abnormal (or pathological) bleeding. Examples include, but are not limited to, disorders of insufficient clotting or hemostasis, such as hemophilia A (a deficiency in fVIII), hemophilia B (a deficiency in fIX), hemophilia C (a deficiency in Factor XI), other clotting factor deficiencies (such as Factor VII or fXIII), abnormal levels of clotting factor inhibitors, platelet disorders, thrombocytopenia, vitamin K deficiency and von Willebrand's disease.

Some clotting disorders are present at birth and in some instances are inherited disorders. Specific examples include, but are not limited to: hemophilia A, hemophilia B, protein C deficiency, and Von Willebrand's disease. Some clotting disorders are developed during certain illnesses (such as vitamin K deficiency, severe liver disease), or treatments (such as use of anticoagulant drugs or prolonged use of antibiotics).

Clotting factor: Includes any protein which promotes proper hemostasis. In one embodiment, a clotting factor is fVIII or fIX, or a variant or fragment thereof which retains its hemostatic activity, for example as measured using an APTT assay or a bleeding time assay. In some embodiments, when administered in a therapeutically effective amount, the clotting factor increases hemostasis in a subject suffering from a clotting disorder, such as hemophilia.

Clotting Factor VIII (fVIII): fVIII is a protein required for the efficient clotting of blood, and functions in coagulation as a cofactor in the activation of factor X by fIX. A concentration of about 100 ng/ml for fVIII in the blood is considered in the normal range. Deficiency of fVIII is associated with hemophilia A, and severe forms of the disease can result when a subject has less than about 1% of the normal amount of fVIII (*i.e.* less than about 1 ng of fVIII per ml of blood). fVIII is synthesized as a 2351 amino acid single chain precursor protein, which is proteolytically processed. The human factor VIII gene (186,000 base-pairs) consists of 26 exons ranging in size from 69 to 3,106 bp and introns as large as 32.4 kilobases (kb). Examples of fVIII nucleic acid and protein sequences are publicly available (for example, see Genbank Accession Nos: K01740, M14113, and E00527). fVIII variants are provided herein that retain fVIII activity for blood clotting but are reduced in size, such as fVIII variants that lack the fVIII B domain. Exemplary fVIII variants include the HSQ and ET3 variants.

Clotting Factor IX (fIX): fIX is a vitamin K-dependent protein required for the efficient clotting of blood, and functions in coagulation as an activator of factor X. A concentration of about 1-5 μg/ml of fIX in the blood is considered in the normal range. Deficiency of fIX is associated with hemophilia B, and severe cases result when the concentration of fIX is less than about 1% of the normal concentration of fIX (*i.e.* less than about 0.01-0.05 μg fIX per ml of blood). fX nucleic acid and protein sequences are publicly available (for example see Kurachi et al., 1982. Proc. Natl. Acad. Sci. U.S.A. 79(21):6461-4; Genbank Accession Nos: J00136, XM045316, K02402, J00137, and M11309.

Codon-optimized: A "codon-optimized" nucleic acid refers to a nucleic acid sequence that has been altered such that the codons are optimal for expression in a particular system (such as a particular species or group of species). For example, a nucleic acid sequence can be optimized for expression in mammalian cells or in a particular mammalian species (such as human cells). Codon optimization does not alter the amino acid sequence of the encoded protein.

The term "liver specific amino acids codons" refers to codons that are differentially utilized-represented in genes highly expressed within the human liver compared to the codon usage of the entire coding region of the human genome. A strategy using a maximum amount of liver specific amino acid codons seeks to avoid codons that are under-represented, *e.g.*, because of low quantities of codon matching tRNA in liver cells resulting in slower protein translation.

Control: A reference standard. In some embodiments, the control is a negative control sample obtained from a healthy patient. In other embodiments, the control is a positive control sample obtained from a patient diagnosed with hemophilia. In still other embodiments, the control is a historical control or standard reference value or range of values (such as a previously tested control sample, such as a group of hemophilia A patients with known prognosis or outcome, or group of samples that represent baseline or normal values).

A difference between a test sample and a control can be an increase or conversely a decrease. The difference can be a qualitative difference or a quantitative difference, for example a statistically significant difference. In some examples, a difference is an increase or decrease, relative to a control, of at least about 5%, such as at least about 10%, at least about 20%, at least about 30%, at least about 40%,

at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 100%, at least about 150%, at least about 200%, at least about 250%, at least about 300%, at least about 350%, at least about 400%, at least about 500%, or greater than 500%.

DNA (deoxyribonucleic acid): DNA is a long chain polymer which comprises the genetic material of most living organisms (some viruses have genes comprising ribonucleic acid (RNA)). The repeating units in DNA polymers are four different nucleotides, each of which comprises one of the four bases, adenine (A), guanine (G), cytosine (C), and thymine (T) bound to a deoxyribose sugar to which a phosphate group is attached. Triplets of nucleotides (referred to as codons) code for each amino acid in a polypeptide, or for a stop signal. The term codon is also used for the corresponding (and complementary) sequences of three nucleotides in the mRNA into which the DNA sequence is transcribed.

Unless otherwise specified, any reference to a DNA molecule is intended to include the reverse complement of that DNA molecule. Except where single-strandedness is required by the text herein, DNA molecules, though written to depict only a single strand, encompass both strands of a double-stranded DNA molecule. Thus, a reference to the nucleic acid molecule that encodes a specific protein, or a fragment thereof, encompasses both the sense strand and its reverse complement. For instance, it is appropriate to generate probes or primers from the reverse complement sequence of the disclosed nucleic acid molecules.

Enhancer: A nucleic acid sequence that increases the rate of transcription by increasing the activity of a promoter.

Flanking: Near or next to, also, including adjoining, for instance in a linear or circular polynucleotide, such as a DNA molecule.

Gene: A nucleic acid sequence, typically a DNA sequence, that comprises control and coding sequences necessary for the transcription of an RNA, whether an mRNA or otherwise. For instance, a gene may comprise a promoter, one or more enhancers or silencers, a nucleic acid sequence that encodes a RNA and/or a polypeptide, downstream regulatory sequences and, possibly, other nucleic acid sequences involved in regulation of the expression of an mRNA.

As is well known in the art, most eukaryotic genes contain both exons and introns. The term "**exon**" refers to a nucleic acid sequence found in genomic DNA that is bioinformatically predicted and/or experimentally confirmed to contribute a contiguous sequence to a mature mRNA transcript. The term "**intron**" refers to a nucleic acid sequence found in genomic DNA that is predicted and/or confirmed not to contribute to a mature mRNA transcript, but rather to be "spliced out" during processing of the transcript.

Gene therapy: The introduction of a heterologous nucleic acid molecule into one or more recipient cells, wherein expression of the heterologous nucleic acid in the recipient cell affects the cell's function and results in a therapeutic effect in a subject. For example, the heterologous nucleic acid molecule may encode a protein, which affects a function of the recipient cell.

Hemophilia: A blood coagulation disorder caused by a deficient clotting factor activity, which decreases hemostasis. Severe forms result when the concentration of clotting factor is less than about 1% of the normal concentration of the clotting factor in a normal subject. In some subjects, hemophilia is due

to a genetic mutation which results in impaired expression of a clotting factor. In others, hemophilia is an auto-immune disorder, referred to as acquired hemophilia, in which the antibodies which are generated against a clotting factor in a subject result in decreased hemostasis.

Hemophilia A results from a deficiency of functional clotting fVIII, while hemophilia B results from a deficiency of functional clotting fIX. These conditions which are due to a genetic mutation are caused by an inherited sex-linked recessive trait with the defective gene located on the X chromosome, and this disease is therefore generally found only in males. The severity of symptoms can vary with this disease, and the severe forms become apparent early on. Bleeding is the hallmark of the disease and typically occurs when a male infant is circumcised. Additional bleeding manifestations make their appearance when the infant becomes mobile. Mild cases may go unnoticed until later in life when they occur in response to surgery or trauma. Internal bleeding may happen anywhere, and bleeding into joints is common.

Hemostasis: Arrest of bleeding blood by blood clot formation. Blood clotting time is the length of time it takes for peripheral blood to clot using an activated partial thromboplastin time assay (APTT) or by measuring bleeding time. In a particular embodiment, the blood clotting time decreases by at least 50%, for example at least 60%, at least 70%, at least 75%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99% or even about 100% (*i.e.* the blood clotting time is similar to what is observed for a normal subject) when compared to the blood clotting time of the subject prior to administration of a therapeutic vector encoding the appropriate clotting factor as described herein. In yet another embodiment, the blood clotting time in the affected subject is corrected to about 50% of a normal subject, to about 75% of a normal subject, to about 90% of a normal subject, for example to about 95%, for example about 100%, after oral administration of a therapeutically effective amount of the appropriate clotting factor. As used herein, "about" refers to plus or minus 5% from a reference value. Thus, about 50% refers to 47.5% to 52.5%.

Intron: A stretch of DNA within a gene that does not contain coding information for a protein. Introns are removed before translation of a messenger RNA.

Inverted terminal repeat (ITR): Symmetrical nucleic acid sequences in the genome of adeno-associated viruses required for efficient replication. ITR sequences are located at each end of the AAV DNA genome. The ITRs serve as the origins of replication for viral DNA synthesis and are essential *cis* components for generating AAV integrating vectors.

Isolated: An "isolated" biological component (such as a nucleic acid molecule, protein, virus or cell) has been substantially separated or purified away from other biological components in the cell or tissue of the organism, or the organism itself, in which the component naturally occurs, such as other chromosomal and extra-chromosomal DNA and RNA, proteins and cells. Nucleic acid molecules and proteins that have been "isolated" include those purified by standard purification methods. The term also embraces nucleic acid molecules and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acid molecules and proteins.

Nucleic acid molecule: A polymeric form of nucleotides, which may include both sense and anti-sense strands of RNA, cDNA, genomic DNA, and synthetic forms and mixed polymers of the above.

A nucleotide refers to a ribonucleotide, deoxynucleotide or a modified form of either type of nucleotide. The term "nucleic acid molecule" as used herein is synonymous with "nucleic acid" and "polynucleotide." A nucleic acid molecule is usually at least 10 bases in length, unless otherwise specified. The term includes single- and double-stranded forms of DNA. A polynucleotide may include either or both naturally occurring and modified nucleotides linked together by naturally occurring and/or non-naturally occurring nucleotide linkages. "cDNA" refers to a DNA that is complementary or identical to an mRNA, in either single stranded or double stranded form. "Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (*i.e.*, rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom.

Nucleotide: This term includes, but is not limited to, a monomer that includes a base linked to a sugar, such as a pyrimidine, purine or synthetic analogs thereof, or a base linked to an amino acid, as in a peptide nucleic acid (PNA). A nucleotide is one monomer in a polynucleotide. A nucleotide sequence refers to the sequence of bases in a polynucleotide.

Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, in the same reading frame.

ORF (open reading frame): A series of nucleotide triplets (codons) coding for amino acids. These sequences are usually translatable into a peptide.

Pharmaceutically acceptable carriers: The pharmaceutically acceptable carriers of use are conventional. *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, PA, 19th Edition, 1995, describes compositions and formulations suitable for pharmaceutical delivery of the disclosed vectors.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (*e.g.*, powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically neutral carriers, pharmaceutical compositions (such as vector compositions) to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate. In particular embodiments, suitable for administration to a subject the carrier may be sterile, and/or suspended or otherwise contained in a unit dosage form containing one or more measured doses of the composition suitable to induce the desired immune response. It may also be accompanied by medications for its use for treatment purposes. The unit dosage form may be, for example, in a sealed vial that contains sterile contents or a syringe for

injection into a subject, or lyophilized for subsequent solubilization and administration or in a solid or controlled release dosage.

Polypeptide: Any chain of amino acids, regardless of length or post-translational modification (*e.g.,* glycosylation or phosphorylation). "Polypeptide" applies to amino acid polymers including naturally occurring amino acid polymers and non-naturally occurring amino acid polymer as well as in which one or more amino acid residue is a non-natural amino acid, for example, an artificial chemical mimetic of a corresponding naturally occurring amino acid. A "residue" refers to an amino acid or amino acid mimetic incorporated in a polypeptide by an amide bond or amide bond mimetic. A polypeptide has an amino terminal (N-terminal) end and a carboxy terminal (C-terminal) end. "Polypeptide" is used interchangeably with peptide or protein, and is used herein to refer to a polymer of amino acid residues.

Preventing, treating or ameliorating a disease: "Preventing" a disease (such as hemophilia) refers to inhibiting the full development of a disease. "Treating" refers to a therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition after it has begun to develop. "Ameliorating" refers to the reduction in the number or severity of signs or symptoms of a disease.

Promoter: A region of DNA that directs/initiates transcription of a nucleic acid (*e.g.* a gene). A promoter includes necessary nucleic acid sequences near the start site of transcription. Typically, promoters are located near the genes they transcribe. A promoter also optionally includes distal enhancer or repressor elements which can be located as much as several thousand base pairs from the start site of transcription. A tissue-specific promoter is a promoter that directs/initiated transcription primarily in a single type of tissue or cell. For example, a **liver-specific promoter** is a promoter that directs/initiates transcription in liver tissue to a substantially greater extent than other tissue types.

Protein: A biological molecule expressed by a gene or other encoding nucleic acid (*e.g.*, a cDNA) and comprised of amino acids.

Purified: The term "purified" does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified peptide, protein, virus, or other active compound is one that is isolated in whole or in part from naturally associated proteins and other contaminants. In certain embodiments, the term "substantially purified" refers to a peptide, protein, virus or other active compound that has been isolated from a cell, cell culture medium, or other crude preparation and subjected to fractionation to remove various components of the initial preparation, such as proteins, cellular debris, and other components.

Recombinant: A recombinant nucleic acid molecule is one that has a sequence that is not naturally occurring, for example, includes one or more nucleic acid substitutions, deletions or insertions, and/or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination can be accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, for example, by genetic engineering techniques.

A recombinant virus is one that includes a genome that includes a recombinant nucleic acid molecule. As used herein, "**recombinant AAV**" refers to an AAV particle in which a recombinant nucleic

acid molecule (such as a recombinant nucleic acid molecule encoding a clotting factor) has been packaged.

A recombinant protein is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. In several embodiments, a recombinant protein is encoded by a heterologous (for example, recombinant) nucleic acid that has been introduced into a host cell, such as a bacterial or eukaryotic cell, or into the genome of a recombinant virus.

Response element (RE): A DNA sequence included in a promoter to which one or more transcription factors can bind to and confer an aspect of control of gene expression.

Sequence identity: The identity or similarity between two or more nucleic acid sequences, or two or more amino acid sequences, is expressed in terms of the identity or similarity between the sequences. Sequence identity can be measured in terms of percentage identity; the higher the percentage, the more identical the sequences are. Sequence similarity can be measured in terms of percentage similarity (which takes into account conservative amino acid substitutions); the higher the percentage, the more similar the sequences are. Homologs or orthologs of nucleic acid or amino acid sequences possess a relatively high degree of sequence identity/similarity when aligned using standard methods. This homology is more significant when the orthologous proteins or cDNAs are derived from species which are more closely related (such as human and mouse sequences), compared to species more distantly related (such as human and *C. elegans* sequences).

Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith & Waterman, *Adv. Appl. Math.* 2:482, 1981; Needleman & Wunsch, *J. Mol. Biol.* 48:443, 1970; Pearson & Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444, 1988; Higgins & Sharp, *Gene*, 73:237-44, 1988; Higgins & Sharp, *CABIOS* 5:151-3, 1989; Corpet *et al.*, *Nuc. Acids Res.* 16:10881-90, 1988; Huang *et al. Computer Appls. in the Biosciences* 8, 155-65, 1992; and Pearson *et al.*, *Meth. Mol. Bio.* 24:307-31, 1994. Altschul *et al.*, *J. Mol. Biol.* 215:403-10, 1990, presents a detailed consideration of sequence alignment methods and homology calculations.

The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, *J. Mol. Biol.* 215:403-10, 1990) is available from several sources, including the National Center for Biological Information (NCBI) and on the internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. Additional information can be found at the NCBI web site.

As used herein, reference to "at least 90% identity" refers to "at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or even 100% identity" to a specified reference sequence.

Subject: Living multi-cellular vertebrate organisms, a category that includes human and non-human mammals.

Synthetic: Produced by artificial means in a laboratory, for example a synthetic nucleic acid can be chemically synthesized in a laboratory.

TATA box: A DNA sequence found in the promoter region of a gene that can be bound by TATA binding protein and transcription factor II D during DNA unwinding and binding by RNA

polymerase II. A TATA box sequence typically includes a TATAAA sequence and often includes additional 3' adenine nucleotides. An exemplary TATA box sequence is provided as nucleotides 108-114 of SEQ ID NO: 4.

Therapeutically effective amount: A quantity of a specified pharmaceutical or therapeutic agent (*e.g.* a recombinant AAV) sufficient to achieve a desired effect in a subject, or in a cell, being treated with the agent. The effective amount of the agent will be dependent on several factors, including, but not limited to the subject or cells being treated, and the manner of administration of the therapeutic composition.

Transcription factor (TF): A protein that binds to specific DNA sequences and thereby controls the transfer (or transcription) of genetic information from DNA to RNA. TFs perform this function alone or with other proteins in a complex, by promoting (as an activator), or blocking (as a repressor) the recruitment of RNA polymerase (the enzyme that performs the transcription of genetic information from DNA to RNA) to specific genes. The specific DNA sequences to which a TF binds is known as a response element (RE) or regulatory element. Other names include cis-element and cis-acting transcriptional regulatory element.

Transcription factors interact with their binding sites using a combination of electrostatic (of which hydrogen bonds are a special case) and Van der Waals forces. Due to the nature of these chemical interactions, most transcription factors bind DNA in a sequence specific manner. However, not all bases in the transcription factor-binding site may actually interact with the transcription factor. In addition, some of these interactions may be weaker than others. Thus, many transcription factors do not bind just one sequence but are capable of binding a subset of closely related sequences, each with a different strength of interaction.

For example, although the consensus binding site for the TATA-binding protein (TBP) is TATAAAA; however, the TBP transcription factor can also bind similar sequences such as TATATAT or TATATAA.

Transcription factors (TFs) are classified based on many aspects. For example, the secondary, tertiary and quaternary structures of the protein structures DNA-binding sequence and properties, the interaction with the double helix of the DNA, and the metal and other binding characteristics. The JASPAR database and TRANSFAC (TRANSFAC® 7.0 Public 2005) are two web-based transcription factor databases, their experimentally-proven binding sites, and regulated genes.

HNF1a: Also called HNF1 Homeobox A or HNF1, the HNF1a protein is a transcription factor required for the expression of several liver specific genes. HNF1a forms a homodimer that binds to particular promoter sequences. Exemplary HNF1a TF binding sites include the "HNF1a" TF binding site provided as nucleotides 1-12 of SEQ ID NO: 4, the "HNF1-1" TF binding site provided as nucleotides 16-23 of SEQ ID NO: 4, and the "HNF1-2" TF binding site provided as nucleotides 48-62 of SEQ ID NO: 4. (See, *e.g.*, PubMed Gene ID NO. 6927; Chi et al., "Diabetes mutations delineate an atypical POU domain in HNF-1alpha," Mol. Cell., 10:1129-1137, 2002; and Rose et al., "Structural basis of dimerization, coactivator recognition and MODY3 mutations in HNF-1alpha," Nat. Struct. Biol. 7:744-748, 2000).

HNF3a: A transcription factor required for the expression of several liver specific genes. HNF3a binds to particular promoter sequences. An exemplary HNF3a TF binding site is provided as nucleotides 39-45 of SEQ ID NO: 4. An exemplary HNF3-2 TF binding site is provided as nucleotides 65-71 of SEQ ID NO: 4. (see, e.g., Laganiere et al., "Location analysis of estrogen receptor alpha target promoters reveals that FOXA1 defines a domain of the estrogen response," Proc. Natl. Acad. Sci. U.S.A. 102:11651-11656, 2005; Williamson et al., "BRCA1 and FOXA1 proteins coregulate the expression of the cell cycle-dependent kinase inhibitor p27(Kip1)," Oncogene 25:1391-1399, 2006; Lupien et al., "FoxA1 translates epigenetic signatures into enhancer-driven lineage-specific transcription," Cell 132:958-970, 2008; Song et al., "Role of Foxa1 in regulation of bcl2 expression during oxidative-stress-induced apoptosis in A549 type II pneumocytes," Cell Stress Chaperones, 14:417-425, 2009); and Malik et al., "Histone deacetylase 7 and FoxA1 in estrogen-mediated repression of RPRM," Mol. Cell. Biol. 30:399-412, 2010).

HNF4: A transcription factor required for the expression of several liver specific genes. HNF4 binds to particular promoter sequences. An exemplary HNF4 TF binding site is provided as nucleotides 26-36 of SEQ ID NO: 4. (See, *e.g.*, Wang et al., " Hepatocyte nuclear factor-4α interacts with other hepatocyte nuclear factors in regulating transthyretin gene expression," FEBS J., 277(19):4066-75, 2010).

HP1: A transcription factor required for the expression of several liver specific genes. HP1 binds to particular promoter sequences. An exemplary HP1 TF binding site is provided as nucleotides 75-87 of SEQ ID NO: 4. (See, *e.g.*, Schorpp et al., "Hepatocyte-specific promoter element HP1 of the Xenopus albumin gene interacts with transcriptional factors of mammalian hepatocytes," J Mol Biol., 202(2):307-20, 1988)

Transcription Start Site: The location where transcription starts at the 5' end of a gene sequence. An exemplary Transcription Start Site is provided as nucleotides 116-146 of SEQ ID NO: 4.

Therapeutically effective amount: The amount of agent, such as a disclosed recombinant AAV vector encoding a clotting factor, that is sufficient to prevent, treat (including prophylaxis), reduce and/or ameliorate the symptoms and/or underlying causes of a disorder or disease, for example to prevent, inhibit, and/or treat hemophilia. For instance, this can be the amount necessary to inhibit or prevent viral replication or to measurably alter outward symptoms of the disease or condition.

In one example, a desired response is to reduce clotting time in a subject (such as a subject with hemophilia), for example as measured using a bleeding time assay. The clotting time does not need to be completely restored to that of normal healthy subjects without hemophilia for the method to be effective. For example, administration of a therapeutically effective amount of a vector (such as a fVIII encoding vector) as disclosed herein can decrease the clotting time (or other symptom of the hemophilia) by a desired amount, for example by at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least 100% or more, as compared to a suitable control.

It is understood that to obtain a therapeutic response to the disease or condition can require multiple administrations of the agent. Thus, a therapeutically effective amount encompasses a fractional dose that contributes in combination with previous or subsequent administrations to attaining a therapeutic outcome in the patient. For example, a therapeutically effective amount of an agent can be administered in a single dose, or in several doses, for example daily, during a course of treatment. However, the therapeutically effective amount can depend on the subject being treated, the severity and type of the condition being treated, and the manner of administration. A unit dosage form of the agent can be packaged in a therapeutic amount, or in multiples of the therapeutic amount, for example, in a vial (*e.g.*, with a pierceable lid) or syringe having sterile components.

Vector: A vector is a nucleic acid molecule allowing insertion of foreign nucleic acid without disrupting the ability of the vector to replicate and/or integrate in a host cell. A vector can include nucleic acid sequences that permit it to replicate in a host cell, such as an origin of replication. A vector can also include one or more selectable marker genes and other genetic elements. An expression vector is a vector that contains the necessary regulatory sequences to allow transcription and translation of inserted gene or genes. In some embodiments herein, the vector is an adeno-associated virus (AAV) vector. In some embodiments, the vector is a gamma-retroviral vector, a lentiviral vector, or an adenoviral vector.

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. "Comprising A or B" means including A, or B, or A and B. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

II. Optimized Promotors for Liver-Directed Transcription

Novel promoters are provided herein for promoting transcription in liver tissue and/or cells. As discussed in Example 2, the new promoters were designed using an iterative approach that ultimately identified several promoter sequences that provide unexpectedly high transcription levels (as assayed by measuring expressed protein activity), and are substantially shorter than prior promoter sequences, such as the HLP promoter sequence.

In some embodiments, a recombinant nucleic acid molecule is provided that comprises a promoter comprising a first response element comprises a set of transcription factor (TF) binding sites, including: a HNF1a TF binding site, a HNF1-1 TF binding site, a HNF4 TF binding site, a HNF3a TF binding site, a HNF1-2 TF binding site, a HNF3-2 TF binding site, a HP1 TF binding site, a TATA box; and a Transcription Start Site. These are the transcription factor binding sites included on the HCB promoter.

In some embodiments, the first response element can comprise a nucleotide sequence that is no more than 160 nucleotides in length (such as no more than 150 nucleotides in length, such as 146 nucleotides in length).

In some embodiments, the HNF1a TF binding site comprises or consists of nucleotides 1-12 of SEQ ID NO: 4; the HNF1-1 TF binding site comprises or consists of nucleotides 16-23 of SEQ ID NO: 4; the HNF4 TF binding site comprises or consists of nucleotides 26-36 of SEQ ID NO: 4; the HNF3a TF binding site comprises or consists of nucleotides 39-45 of SEQ ID NO: 4; the HNF1-2 TF binding site comprises or consists of nucleotides 48-62 of SEQ ID NO: 4; the HNF3-2 TF binding site comprises or consists of nucleotides 65-71 of SEQ ID NO: 4; the HP1 TF binding site comprises or consists of nucleotides 75-87 of SEQ ID NO: 4; the TATA box comprises or consists of nucleotides 108-114 of SEQ ID NO: 4; and/or the Transcription Start Site (TSS) comprises or consists of nucleotides 116-146 of SEQ ID NO: 4.

In some embodiments, the first response element comprises, from 5' to 3', the HNF1a TF binding site, the HNF1-1 TF binding site, the HNF-4 TF binding site, the HNF3a TF binding site, the HNF1-2 TF binding site, the HNF3-2 TF binding site, the HP1 TF binding site, the TATA box, and the Transcription Start Site (TSS).

In some embodiments, the recombinant nucleic acid molecule comprises a promoter comprising or consisting of the nucleic acid sequence set forth as SEQ ID NO: 4 (HCB), or a sequence at least 90% (such as at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 98%, or at least 99%) identical thereto.

In some embodiments, the recombinant nucleic acid molecule can comprise a promoter comprising the first response element as discussed above, and further comprising a second response element.

In some embodiments, the second response element can comprise an HSh response element. For example, a HSh response element comprising or consisting of the nucleotide sequence set forth as SEQ ID NO: 111, or a sequence at least 90% (such as at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 98%, or at least 99%) identical thereto.

In some embodiments, the second response element can comprise a 5'HS response element. For example, a 5'HS response element comprising or consisting of the nucleotide sequence set forth as nucleotides 6-32 of SEQ ID NO: 111, or a sequence at least 90% (such as at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 98%, or at least 99%) identical thereto.

In some embodiments, the second response element can comprise a 3'HS response element. For example, a 3'HS response element comprising or consisting of the nucleotide sequence set forth as nucleotides 44-68 of SEQ ID NO: 111, or a sequence at least 90% (such as at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 98%, or at least 99%) identical thereto.

In some embodiments, the recombinant nucleic acid molecule comprises a promoter comprising or consisting of the nucleic acid sequence set forth as SEQ ID NO: 102 (HSh-HCB), or a sequence at least 90% (such as at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 98%, or at least 99%) identical thereto.

In some embodiments, the recombinant nucleic acid molecule comprises a promoter comprising or consisting of the nucleic acid sequence set forth as SEQ ID NO: 104 (5'HSh-HCB), or a sequence at least 90% (such as at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 98%, or at least 99%) identical thereto.

In some embodiments, the recombinant nucleic acid molecule comprises a promoter comprising or consisting of the nucleic acid sequence set forth as SEQ ID NO: 103 (3'HSh-HCB),

In some embodiments, the recombinant nucleic acid molecule comprises a promoter comprising or consisting of the nucleic acid sequence set forth as SEQ ID NO: 7 (ABP-HP1-God-TSS), or a sequence at least 90% (such as at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 98%, or at least 99%) identical thereto.

In some embodiments, the recombinant nucleic acid molecule comprises a promoter comprising or consisting of the nucleic acid sequence set forth as SEQ ID NO: 105 (HSh-SynO-TSS), or a sequence at least 90% (such as at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 98%, or at least 99%) identical thereto.

In some embodiments, the recombinant nucleic acid molecule comprises a promoter comprising or consisting of the nucleic acid sequence set forth as SEQ ID NO: 106 (sHS-SynO-TSS), or a sequence at least 90% (such as at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 98%, or at least 99%) identical thereto.

In some embodiments, the recombinant nucleic acid molecule comprises a promoter comprising or consisting of the nucleic acid sequence set forth as SEQ ID NO: 107 (Agro), or a sequence at least 90% (such as at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 98%, or at least 99%) identical thereto.

In some embodiments, the recombinant nucleic acid molecule comprises a promoter comprising or consisting of the nucleic acid sequence set forth as SEQ ID NO: 108 (HS-SynO-TSS), or a sequence at least 90% (such as at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 98%, or at least 99%) identical thereto.

In some embodiments, the recombinant nucleic acid molecule comprises a promoter comprising or consisting of the nucleic acid sequence set forth as SEQ ID NO: 112 (HNF1-ShortABPExact-SynO-TSS-Int), or a sequence at least 90% (such as at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 98%, or at least 99%) identical thereto.

In some embodiments, the recombinant nucleic acid molecule comprises a promoter comprising or consisting of the nucleic acid sequence set forth as SEQ ID NO: 5 (shortABP-HP1-God-TSS), or a sequence at least 90% (such as at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 98%, or at least 99%) identical thereto.

In some embodiments, the recombinant nucleic acid molecule comprises a promoter comprising or consisting of the nucleic acid sequence set forth as SEQ ID NO: 7 (ABP-HP1-God-TSS), or a sequence at least 90% (such as at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 98%, or at least 99%) identical thereto.

The disclosed promoters can be utilized in any situation where liver-specific transcription is desired. In several embodiments, any one of the disclosed promoters can be included on a vector (such as an AAV vector) for gene therapy methods where liver-specific expression of a transgene is desired, such as liver specific expression of a clotting factor as disclosed herein.

III. Recombinant Nucleic Acid Molecules Encoding Clotting factors

The blood clotting system is a proteolytic cascade. Blood clotting factors are present in the plasma as a zymogen, in other words in an inactive form, which on activation undergoes proteolytic cleavage to release the active factor form the precursor molecule. The ultimate goal is to produce thrombin. Thrombin converts fibrinogen into fibrin, which forms a clot.

Factor X is the first molecule of the common pathway and is activated by a complex of molecules containing activated fIX, fVIII, calcium, and phospholipids which are on the platelet surface. FVIII is activated by thrombin, and it facilitates the activation of factor X by fIXa. FVIII, contains multiple domains (A1-A2-B-ap-A3-C1-C2) and circulates in blood in an inactivated form bound to von Willebrand factor (VWF). The C2 domain is involved with fVIII binding to VWF. Thrombin cleaves fVIII causing dissociation with VWF ultimately leading to fibrin formation through fIX. Congenital hemophilia A is associated with genetic mutations in the fVIII gene and results in impaired clotting due to lower than normal levels of circulating fVIII. Hemophilia B is similarly associated with genetic mutations in the fIX gene.

FVIII domain boundaries refer to the human fVIII amino acid sequence numbering as follows; residues 1-19 (Signal Sequence), 20–391 (A1), 392–759 (A2), 760-1667 (B), 1668–1708 (ap), 1709–2038 (A3), 2039–2191 (C1) and 2192–2351 (C2) (see Gitschier et al., Nature, 1984, 312, 326–330) of SEQ ID NO: 1:

MQIELSTCFFLCLLRFCFSATRRYYLGAVELSWDYMQSDLGELPVDARFPPRVPKSFPFNTSVVYKKTLFVEFTDHLF
NIAKPRPPWMGLLGPTIQAEVYDTVVITLKNMASHPVSLHAVGVSYWKASEGAEYDDQTSQREKEDDKVFPGGSHTYV
WQVLKENGPMASDPLCLTYSYLSHVDLVKDLNSGLIGALLVCREGSLAKEKTQTLHKFILLFAVFDEGKSWHSETKNS
LMQDRDAASARAWPKMHTVNGYVNRSLPGLIGCHRKSVYWHVIGMGTTPEVHSIFLEGHTFLVRNHRQASLEISPITF
LTAQTLLMDLGQFLLFCHISSHQHDGMEAYVKVDSCPEEPQLRMKNNEEAEDYDDDLTDSEMDVVRFDDDNSPSFIQI
RSVAKKHPKTWVHYIAAEEEDWDYAPLVLAPDDRSYKSQYLNNGPQRIGRKYKKVRFMAYTDETFKTREAIQHESGIL
GPLLYGEVGDTLLIIFKNQASRPYNIYPHGITDVRPLYSRRLPKGVKHLKDFPILPGEIFKYKWTVTVEDGPTKSDPR
CLTRYYSSFVNMERDLASGLIGPLLICYKESVDQRGNQIMSDKRNVILFSVFDENRSWYLTENIQRFLPNPAGVQLED
PEFQASNIMHSINGYVFDSLQLSVCLHEVAYWYILSIGAQTDFLSVFFSGYTFKHKMVYEDTLTLFPFSGETVFMSME
NPGLWILGCHNSDFRNRGMTALLKVSSCDKNTGDYYEDSYEDISAYLLSKNNAIEPRSFSQNSRHPSTRQKQFNATTI
PENDIEKTDPWFAHRTPMPKIQNVSSSDLLMLLRQSPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRP
QLHHSGDMVFTPESGLQLRLNEKLGTTAATELKKLDFKVSSTSNNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQL
DTTLFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSSTESGRLFKGKRAHGPALLTKDNALFKVSI
SLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTPLIHDRMLMDKNATALRLNHMSNKTTSSKN
MEMVQQKKEGPIPPDAQNPDMSFFKMLFLPESARWIQRTHGKNSLNSGQGPSPKQLVSLGPEKSVEGQNFLSEKNKVV
VGKGEFTKDVGLKEMVFPSSRNLFLTNLDNLHENNTHNQEKKIQEEIEKKETLIQENVVLPQIHTVTGTKNFMKNLFL
LSTRQNVEGSYDGAYAPVLQDFRSLNDSTNRTKKHTAHFSKKGEEENLEGLGNQTKQIVEKYACTTRISPNTSQQNFV
TQRSKRALKQFRLPLEETELEKRIIVDDTSTQWSKNMKHLTPSTLTQIDYNEKEKGAITQSPLSDCLTRSHSIPQANR
SPLPIAKVSSFPSIRPIYLTRVLFQDNSSHLPAASYRKKDSGVQESSHFLQGAKKNNLSLAILTLEMTGDQREVGSLG
TSATNSVTYKKVENTVLPKPDLPKTSGKVELLPKVHIYQKDLFPTETSNGSPGHLDLVEGSLLQGTEGAIKWNEANRP
GKVPFLRVATESSAKTPSKLLDPLAWDNHYGTQIPKEEWKSQEKSPEKTAFKKKDTILSLNACESNHAIAAINEGQNK
PEIEVTWAKQGRTERLCSQNPPVLKRHQREITRTTLQSDQEEIDYDDTISVEMKKEDFDIYDEDENQSPRSFQKKTRH
YFIAAVERLWDYGMSSSPHVLRNRAQSGSVPQFKKVVFQEFTDGSFTQPLYRGELNEHLGLLGPYIRAEVEDNIMVTF
RNQASRPYSFYSSLISYEEDQRQGAEPRKNFVKPNETKTYFWKVQHHMAPTKDEFDCKAWAYFSDVDLEKDVHSGLIG
PLLVCHTNTLNPAHGRQVTVQEFALFFTIFDETKSWYFTENMERNCRAPCNIQMEDPTFKENYRFHAINGYIMDTLPG
LVMAQDQRIRWYLLSMGSNENIHSIHFSGHVFTVRKKEEYKMALYNLYPGVFETVEMLPSKAGIWRVECLIGEHLHAG

MSTLFLVYSNKCQTPLGMASGHIRDFQITASGQYGQWAPKLARLHYSGSINAWSTKEPFSWIKVDLLAPMIIHGIKTQ
GARQKFSSLYISQFIIMYSLDGKKWQTYRGNSTGTLMVFFGNVDSSGIKHNIFNPPIIARYIRLHPTHYSIRSTLRME
LMGCDLNSCSMPLGMESKAISDAQITASSYFTNMFATWSPSKARLHLQGRSNAWRPQVNNPKEWLQVDFQKTMKVTGV
TTQGVKSLLTSMYVKEFLISSSQDGHQWTLFFQNGKVKVFQGNQDSFTPVVNSLDPPLLTRYLRIHPQSWVHQIALRM
EVLGCEAQDLY

As discussed in Example 1, the cDNA nucleotide sequences coding for fVIII variants ET3 and HSQ were improved by implementing a codon usage bias specific for the human liver cell as compared to naturally occurring nucleotide sequence coding for the corresponding non-codon optimized sequence for a human. Additional changes were also made to improve translation efficacy, such as optimization of GC content, mRNA secondary structure, premature PolyA sites, RNA instability motif, stable free energy of mRNA, internal chi sites, ribosomal binding sites, cryptic splicing sites, negative CpG islands, SD sequence, TATA boxes, and cyptic terminal signals.

In addition, CpG DNA motifs were removed because they may lead to gene methylation and silencing. See Bird, DNA methylation and the frequency of CpG in animal DNA, 1980, Nucleic Acids Res, 8: 1499–1504. Codons were substituted with the most highly used human/ liver alternative that did not result in the formation of a 5'-CG-3' dinucleotide in the sequence. CpG removal can also reduce any immune response to a vector including the modified transgene, enhancing the safety and efficacy of the vector. See J Clin Invest. 2013, 123(7):2994-3001, entitled "CpG-depleted adeno-associated virus vectors evade immune detection."

ET3 is a B domain deleted (BDD) fVIII hybrid that contains human and porcine domains, *i.e.*, sequence (A1 and A3 porcine, see FIGs. 1A and 1B) with a linker in the deleted B domain. ET3 utilizes a 24 amino acid porcine sequence-derived OL linker sequence, *i.e.*, porcine-derived sequence SFAQNSRPPSASAPKPPVLRRHQR (SEQ ID NO: 23). HSQ is a human fVIII variant wherein the BDD human fVIII protein is substituted with a 14 amino acid human-derived SQ linker SFSQNPPVLKRHQR (SEQ ID NO: 22). HSQ amino acid sequence is provided as SEQ ID NO: 3. Both HSQ and ET3 contain the RHQR (SEQ ID NO: 24) recognition sequence for PACE/furin processing sequence for the B-domain.

As discussed in Example 1, the nucleotide sequence encoding ET3 was codon-optimized for expression in human liver. An exemplary liver codon optimized ET3 sequence is provided as SEQ ID NO: 12. In some embodiments, a recombinant nucleic acid molecule is provided comprising the nucleotide sequence set forth as SEQ ID NO: 12, or a sequence at least 90% (such as at least 95%) identical thereto. Further, CpG motifs within the codon-optimized ET3 sequence were removed, to provide the CpG deleted, liver codon optimized ET3 sequence set forth as SEQ ID NO: 11. In some embodiments, a recombinant nucleic acid molecule is provided comprising the nucleotide sequence set forth as SEQ ID NO: 11, or a sequence at least 90% (such as at least 95%) identical thereto.

As discussed in Example 1, the nucleotide sequence encoding HSQ was codon-optimized for expression in human liver. Further, CpG motifs within the codon-optimized HSQ sequence were removed, to provide the CpG deleted, liver codon optimized HSQ sequence set forth as SEQ ID NO: 2. In some embodiments, a recombinant nucleic acid molecule is provided comprising the nucleotide sequence set forth as SEQ ID NO: 2, or a sequence at least 90% (such as at least 95%) identical thereto.

Additionally, the nucleotide sequences encoding ET3 and HSQ were optimized for expression in myeloid cells. An exemplary CpG deleted, myeloid codon-optimized ET3 sequence is provided as SEQ ID NO: 125. In some embodiments, a recombinant nucleic acid molecule is provided comprising the nucleotide sequence set forth as SEQ ID NO: 125, or a sequence at least 90% (such as at least 95%) identical thereto. An exemplary CpG deleted, myeloid codon-optimized HSQ sequence is provided as SEQ ID NO: 126. In some embodiments, a recombinant nucleic acid molecule is provided comprising the nucleotide sequence set forth as SEQ ID NO: 126, or a sequence at least 90% (such as at least 95%) identical thereto.

In additional embodiments, fIX coding sequence variants are provided that are designed for high levels of expression when the transgene is expressed from the liver, which is the target tissue of many fIX-targeted gene therapy strategies. To create this coding sequence, one utilizes a liver-directed codon optimization strategy.

As discussed in Example 1, the nucleotide sequence coding for fIX was optimized by implementing a codon usage bias specific for the human liver cell as compared to naturally occurring nucleotide sequence coding for the corresponding non-codon optimized sequence for a human. Additional changes were also made to improve Translation efficacy, such as optimization of GC content, mRNA secondary structure, premature PolyA sites, RNA instability motif, stable free energy of mRNA, internal chi sites, ribosomal binding sites, cryptic splicing sites, negative CpG islands, SD sequence, TATA boxes, and cyptic terminal signals.

In addition to adjusting the codon usage bias, the resulting sequences are further modified to remove CpG motifs which may inhibit efficient expression of the transgene. Further, in some embodiments, the recombinant fIX nucleic acid molecule can encode fIX with the K5A mutation (Darrel Stafford collagen binding mutation, Gui et al. Blood. 2002, 100(1):153-8). In certain embodiments, the recombinant fIX nucleic acid molecule can encode fIX with the R338L mutation (Padua mutation), which is a naturally occurring gain of function mutation that has been shown to improve the specific activity of fIX by 8-fold. Sequence variants were additionally created to reflect two major polymorphisms of fIX at residue 148, including alanine or threonine. In some embodiments, these fIX sequences may be grafted into liver-directed AAV as either single-stranded or self-complimentary double stranded transgene designs.

Exemplary recombinant nucleic acid sequences encoding fVIII or fIX proteins, or variants thereof, that are modified for tissue-specific expression are discussed in Example 1.

SEQ ID NO: 12 provides an exemplary liver codon optimized ET3 sequence. In some embodiments, a recombinant nucleic acid molecule is provided that comprises or consists of the nucleic acid sequence set forth as SEQ ID NO: 12, or a sequence at least 90% (such as at least 95%) identical thereto.

SEQ ID NO: 11 provides an exemplary CpG deleted, liver codon optimized ET3 sequence. In some embodiments, a recombinant nucleic acid molecule is provided that comprises or consists of the nucleic acid sequence set forth as SEQ ID NO: 11, or a sequence at least 90% (such as at least 95%) identical thereto.

SEQ ID NO: 2 provides an exemplary CpG deleted, liver codon optimized HSQ sequence. In some embodiments, a recombinant nucleic acid molecule is provided that comprises or consists of the nucleic acid sequence set forth as SEQ ID NO: 2, or a sequence at least 90% (such as at least 95%) identical thereto.

SEQ ID NO: 125 provides an exemplary CpG deleted, myeloid codon optimized ET3 sequence. In some embodiments, a recombinant nucleic acid molecule is provided that comprises or consists of the nucleic acid sequence set forth as SEQ ID NO: 125, or a sequence at least 90% (such as at least 95%) identical thereto.

SEQ ID NO: 126 provides an exemplary CpG deleted, myeloid codon optimized HSQ sequence. In some embodiments, a recombinant nucleic acid molecule is provided that comprises or consists of the nucleic acid sequence set forth as SEQ ID NO: 125, or a sequence at least 90% (such as at least 95%) identical thereto.

Exemplary recombinant nucleic acid sequences encoding fIX proteins, or variants thereof, that are modified for tissue-specific expression are discussed in Example 1.

SEQ ID NO: 124 provides an exemplary liver codon optimized fIX sequence with Padua/Malmo mutations and no CpG. In some embodiments, a recombinant nucleic acid molecule is provided that comprises or consists of the nucleic acid sequence set forth as SEQ ID NO: 124, or a sequence at least 90% (such as at least 95%) identical thereto.

SEQ ID NO: 8 provides an exemplary liver-codon optimized fIX sequence with no CpG and encoding A582 modifications. In some embodiments, a recombinant nucleic acid molecule is provided that comprises or consists of the nucleic acid sequence set forth as SEQ ID NO: 8, or a sequence at least 90% (such as at least 95%) identical thereto.

SEQ ID NO: 9 provides an exemplary liver codon optimized fIX sequence with no CpG and including Padua and A582 modifications. In some embodiments, a recombinant nucleic acid molecule is provided that comprises or consists of the nucleic acid sequence set forth as SEQ ID NO: 9, or a sequence at least 90% (such as at least 95%) identical thereto.

SEQ ID NO: 10 provides an exemplary liver codon optimized fIX sequence with Padua/Malmo mutations and no CpG. In some embodiments, a recombinant nucleic acid molecule is provided that comprises or consists of the nucleic acid sequence set forth as SEQ ID NO:10, or a sequence at least 90% (such as at least 95%) identical thereto.

SEQ ID NO: 127 provides an exemplary human codon optimized fIX sequence with Padua/Malmo mutations and no CpG. In some embodiments, a recombinant nucleic acid molecule is provided that comprises or consists of the nucleic acid sequence set forth as SEQ ID NO:127, or a sequence at least 90% (such as at least 95%) identical thereto.

Any of the above discussed recombinant nucleic acid molecules encoding a fVIII or fIX protein, or variant thereof, can be included in an vector (such as a AAV vector) as described herein for embodiments where expression of a fVIII or FIX protein or variant thereof is of interest.

In some embodiments, an isolated protein is provided comprising an amino acid sequence encoded by one of SEQ ID NOs: 8, 9, or 10, such as the amino acid sequences set forth as SEQ ID NOs:

17-18 below. In some embodiments, an isolated protein is provided comprising an amino acid sequence set forth as SEQ ID NO: 17, or an amino acid sequence at least 90% (such as at least 95%) identical thereto having fIX activity. In some embodiments, an isolated protein is provided comprising an amino acid sequence set forth as SEQ ID NO: 18, or an amino acid sequence at least 90% (such as at least 95%) identical thereto having fIX activity. In some embodiments, an isolated protein is provided comprising an amino acid sequence set forth as SEQ ID NO: 19, or an amino acid sequence at least 90% (such as at least 95%) identical thereto having fIX activity.

SEQ ID NO: 8 encodes the amino acid sequence set forth as SEQ ID NO: 17:

MQRVNMIMAESPGLITICLLGYLLSAECTVFLDHENANKILNRPKRYNSGKLEEFVQGNLERECMEEKCSFEEAREVF
ENTERTTEFWKQYVDGDQCESNPCLNGGSCKDDINSYECWCPFGFEGKNCELDVTCNIKNGRCEQFCKNSADNKVVCS
CTEGYRLAENQKSCEPAVPFPCGRVSVSQTSKLTRAEAVFPDVDYVNSTEAETILDNITQSTQSFNDFTRVVGGEDAK
PGQFPWQVVLNGKVDAFCGGSIVNEKWIVTAAHCVETGVKITVVAGEHNIEETEHTEQKRNVIRIIPHHNYNAAINKY
NHDIALLELDEPLVLNSYVTPICIADKEYTNIFLKFGSGYVSGWGRVFHKGRSALVLQYLRVPLVDRATCLRSTKFTI
YNNMFCAGFHEGGRDSCQGDSGGPHVTEVEGTSFLTGIISWGEECAMKGKYGIYTKVSRYVNWIKEKTKLT

SEQ ID NO: 9 encodes the amino acid sequence set forth as SEQ ID NO: 18:

MQRVNMIMAESPGLITICLLGYLLSAECTVFLDHENANKILNRPKRYNSGKLEEFVQGNLERECMEEKCSFEEAREVF
ENTERTTEFWKQYVDGDQCESNPCLNGGSCKDDINSYECWCPFGFEGKNCELDVTCNIKNGRCEQFCKNSADNKVVCS
CTEGYRLAENQKSCEPAVPFPCGRVSVSQTSKLTRAEAVFPDVDYVNSTEAETILDNITQSTQSFNDFTRVVGGEDAK
PGQFPWQVVLNGKVDAFCGGSIVNEKWIVTAAHCVETGVKITVVAGEHNIEETEHTEQKRNVIRIIPHHNYNAAINKY
NHDIALLELDEPLVLNSYVTPICIADKEYTNIFLKFGSGYVSGWGRVFHKGRSALVLQYLRVPLVDRATCLLSTKFTI
YNNMFCAGFHEGGRDSCQGDSGGPHVTEVEGTSFLTGIISWGEECAMKGKYGIYTKVSRYVNWIKEKTKLT

SEQ ID NO: 10 encodes the amino acid sequence set forth as SEQ ID NO: 19:

MQRVNMIMAESPGLITICLLGYLLSAECTVFLDHENANKILNRPKRYNSGKLEEFVQGNLERECMEEKCSFEEAREVF
ENTERTTEFWKQYVDGDQCESNPCLNGGSCKDDINSYECWCPFGFEGKNCELDVTCNIKNGRCEQFCKNSADNKVVCS
CTEGYRLAENQKSCEPAVPFPCGRVSVSQTSKLTRAETVFPDVDYVNSTEAETILDNITQSTQSFNDFTRVVGGEDAK
PGQFPWQVVLNGKVDAFCGGSIVNEKWIVTAAHCVETGVKITVVAGEHNIEETEHTEQKRNVIRIIPHHNYNAAINKY
NHDIALLELDEPLVLNSYVTPICIADKEYTNIFLKFGSGYVSGWGRVFHKGRSALVLQYLRVPLVDRATCLLSTKFTI
YNNMFCAGFHEGGRDSCQGDSGGPHVTEVEGTSFLTGIISWGEECAMKGKYGIYTKVSRYVNWIKEKTKLT

Exemplary nucleic acids can be prepared by cloning techniques, or can be generated synthetically. Examples of appropriate cloning and sequencing techniques, and instructions sufficient to direct persons of skill through many cloning exercises are known (see, *e.g.*, Sambrook *et al.* (Molecular Cloning: A Laboratory Manual, 4th ed, Cold Spring Harbor, New York, 2012) and Ausubel *et al.* (In Current Protocols in Molecular Biology, John Wiley & Sons, New York, through supplement 104, 2013). Product information from manufacturers of biological reagents and experimental equipment also provide useful information. Such manufacturers include the SIGMA Chemical Company (Saint Louis, MO), R&D Systems (Minneapolis, MN), Pharmacia Amersham (Piscataway, NJ), CLONTECH Laboratories, Inc. (Palo Alto, CA), Chem Genes Corp., Aldrich Chemical Company (Milwaukee, WI), Glen Research, Inc., GIBCO BRL Life Technologies, Inc. (Gaithersburg, MD), Fluka Chemica-Biochemika Analytika (Fluka Chemie AG, Buchs, Switzerland), Invitrogen (Carlsbad, CA), and Applied Biosystems (Foster City, CA), as well as many other commercial sources known to one of skill.

Nucleic acids can also be prepared by amplification methods. Amplification methods include polymerase chain reaction (PCR), the ligase chain reaction (LCR), the transcription-based amplification

system (TAS), the self-sustained sequence replication system (3SR). A wide variety of cloning methods, host cells, and *in vitro* amplification methodologies are well known to persons of skill.

IV. Recombinant Vectors and Gene Therapy Applications

The nucleic acid and promotor sequences disclosed herein are useful in production of vectors (such as rAAV vectors), and are also useful as antisense delivery vectors, gene therapy vectors, or vaccine vectors. In certain embodiments, the disclosure provides for gene delivery vectors, and host cells which contain the nucleic acid sequences disclosed herein. In some embodiments, the selected vector may be delivered to a subject by any suitable method, including intravenous injection, ex-vivo transduction, transfection, electroporation, liposome delivery, membrane fusion techniques, high velocity DNA-coated pellets, viral infection, or protoplast fusion, to introduce a transgene into the subject.

In certain embodiments, the disclosure relates to virus particle, *e.g.*, capsids, containing the nucleic acid sequences encoding promotors and proteins disclosed herein. The virus particles, capsids, and recombinant vectors are useful in delivery of a heterologous gene or other nucleic acid sequences to a target cell. The nucleic acids may be readily utilized in a variety of vector systems, capsids, and host cells. In certain embodiments, the nucleic acids are in vectors contained within a capsid comprising cap proteins, including AAV capsid proteins vp1, vp2, vp3 and hypervariable regions.

In certain embodiments, the nucleic acids disclosed herein may be a part of any genetic element (vector) which may be delivered to a host cell, *e.g.*, naked DNA, a plasmid, phage, transposon, cosmid, episome, a protein in a non-viral delivery vehicle (*e.g.*, a lipid-based carrier), virus, etc. which transfer the sequences carried thereon.

In certain embodiments, a vector may be a lentivirus based (containing lentiviral genes or sequences) vector, *e.g.*, having nucleic acid sequences derived from VSVG or GP64 pseudotypes or both. In certain embodiments, the nucleic acid sequences derived from VSVG or GP64 pseudotypes may be at least one or two or more genes or gene fragments of more than 1000, 500, 400, 300, 200, 100, 50, or 25 continuous nucleotides or nucleotides sequences with greater than 50, 60, 70, 80, 90, 95 or 99 % identity to the gene or fragment.

In some embodiments, a method of inducing blood clotting in a subject in need thereof is provided. The method comprises administering to the subject a therapeutically effective amount of a vector (such as an AAV vector) encoding a clotting factor as described herein. In some embodiments, the subject is a subject with a clotting disorder, such as hemophilia A or hemophilia B. In some embodiments, the clotting disorder is hemophilia A and the subject is administered a vector comprising a nucleic acid molecule encoding a protein with fVIII activity. In other embodiments, the clotting disorder is hemophilia B and the subject is administered a vector comprising a nucleic acid molecule encoding a protein with fIX activity.

In some embodiments, the nucleic acid and promotor sequences disclosed herein are useful in production of AAV vectors. AAV belongs to the family *Parvoviridae* and the genus *Dependovirus*. AAV is a small, non-enveloped virus that packages a linear, single-stranded DNA genome. Both sense and antisense strands of AAV DNA are packaged into AAV capsids with equal frequency.

The AAV genome is characterized by two inverted terminal repeats (ITRs) that flank two open reading frames (ORFs). In the AAV2 genome, for example, the first 125 nucleotides of the ITR are a palindrome, which folds upon itself to maximize base pairing and forms a T-shaped hairpin structure. The other 20 bases of the ITR, called the D sequence, remain unpaired. The ITRs are *cis*-acting sequences important for AAV DNA replication; the ITR is the origin of replication and serves as a primer for second-strand synthesis by DNA polymerase. The double-stranded DNA formed during this synthesis, which is called replicating-form monomer, is used for a second round of self-priming replication and forms a replicating-form dimer. These double-stranded intermediates are processed via a strand displacement mechanism, resulting in single-stranded DNA used for packaging and double-stranded DNA used for transcription. Located within the ITR are the Rep binding elements and a terminal resolution site (TRS). These features are used by the viral regulatory protein Rep during AAV replication to process the double-stranded intermediates. In addition to their role in AAV replication, the ITR is also essential for AAV genome packaging, transcription, negative regulation under non-permissive conditions, and site-specific integration (Daya and Berns, *Clin Microbiol Rev* 21(4):583-593, 2008).

The left ORF of AAV contains the Rep gene, which encodes four proteins – Rep78, Rep 68, Rep52 and Rep40. The right ORF contains the Cap gene, which produces three viral capsid proteins (VP1, VP2 and VP3). The AAV capsid contains 60 viral capsid proteins arranged into an icosahedral symmetry. VP1, VP2 and VP3 are present in a 1:1:10 molar ratio (Daya and Berns, *Clin Microbiol Rev* 21(4):583-593, 2008).

AAV vectors typically contain a transgene expression cassette between the ITRs that replaces the rep and cap genes. Vector particles are produced by the co-transfection of cells with a plasmid containing the vector genome and a packaging/helper construct that expresses the rep and cap proteins in trans. During infection, AAV vector genomes enter the cell nucleus and can persist in multiple molecular states. One common outcome is the conversion of the AAV genome to a double-stranded circular episome by second-strand synthesis or complementary strand pairing.

In the context of AAV vectors, the disclosed vectors typically have a recombinant genome comprising the following structure:

(5'AAV ITR) - (promoter) - (transgene) - (3'AAV ITR)

As discussed above, these recombinant AAV vectors contain a transgene expression cassette between the ITRs that replaces the rep and cap genes. Vector particles are produced, for example, by the co-transfection of cells with a plasmid containing the recombinant vector genome and a packaging/helper construct that expresses the rep and cap proteins in trans. For example, in some embodiments, the recombinant AAV vector can have a genome with a structure set forth as one of:

(5'AAV ITR) - (HCB) - (transgene) - (3'AAV ITR)
(5'AAV ITR) - (HCB) - (fVIII) - (3'AAV ITR)
(5'AAV ITR) - (HCB) - (fVIII-B-domain deleted) - (3'AAV ITR)
(5'AAV ITR) - (HCB) - (ET3) - (3'AAV ITR)

(5'AAV ITR) - (HCB) - (ET3, Seq_12) - (3'AAV ITR)
(5'AAV ITR) - (HCB) - (ET3, Seq_11) - (3'AAV ITR)
(5'AAV ITR) - (HCB) - (HSQ) - (3'AAV ITR)
(5'AAV ITR) - (HCB) - (HSQ, Seq_2) - (3'AAV ITR)
(5'AAV ITR) - (HCB) - (fIX) - (3'AAV ITR)
(5'AAV ITR) - (HCB) - (fIX, Seq_124) - (3'AAV ITR)
(5'AAV ITR) - (HCB) - (fIX, Seq_8) - (3'AAV ITR)
(5'AAV ITR) - (HCB) - (fIX, Seq_9) - (3'AAV ITR)
(5'AAV ITR) - (HCB) - (fIX, Seq_10) - (3'AAV ITR)

(5'AAV ITR) - (HSh-HCB) - (transgene) - (3'AAV ITR)
(5'AAV ITR) - (HSh-HCB) - (fVIII) - (3'AAV ITR)
(5'AAV ITR) - (HSh-HCB) - (fVIII-B-domain deleted) - (3'AAV ITR)
(5'AAV ITR) - (HSh-HCB) - (ET3) - (3'AAV ITR)
(5'AAV ITR) - (HSh-HCB) - (ET3, Seq_12) - (3'AAV ITR)
(5'AAV ITR) - (HSh-HCB) - (ET3, Seq_11) - (3'AAV ITR)
(5'AAV ITR) - (HSh-HCB) - (HSQ) - (3'AAV ITR)
(5'AAV ITR) - (HSh-HCB) - (HSQ, Seq_2) - (3'AAV ITR)
(5'AAV ITR) - (HSh-HCB) - (fIX) - (3'AAV ITR)
(5'AAV ITR) - (HSh-HCB) - (fIX, Seq_124) - (3'AAV ITR)
(5'AAV ITR) - (HSh-HCB) - (fIX, Seq_8) - (3'AAV ITR)
(5'AAV ITR) - (HSh-HCB) - (fIX, Seq_9) - (3'AAV ITR)
(5'AAV ITR) - (HSh-HCB) - (fIX, Seq_10) - (3'AAV ITR)

(5'AAV ITR) - (5'HSh-HCB) - (transgene) - (3'AAV ITR)
(5'AAV ITR) - (5'HSh-HCB) - (fVIII-B-domain deleted) - (3'AAV ITR)
(5'AAV ITR) - (5'HSh-HCB) - (ET3) - (3'AAV ITR)
(5'AAV ITR) - (5'HSh-HCB) - (ET3, Seq_12) - (3'AAV ITR)
(5'AAV ITR) - (5'HSh-HCB) - (ET3, Seq_11) - (3'AAV ITR)
(5'AAV ITR) - (5'HSh-HCB) - (HSQ) - (3'AAV ITR)
(5'AAV ITR) - (5'HSh-HCB) - (HSQ, Seq_2) - (3'AAV ITR)
(5'AAV ITR) - (5'HSh-HCB) - (fIX) - (3'AAV ITR)
(5'AAV ITR) - (5'HSh-HCB) - (fIX, Seq_124) - (3'AAV ITR)
(5'AAV ITR) - (5'HSh-HCB) - (fIX, Seq_8) - (3'AAV ITR)
(5'AAV ITR) - (5'HSh-HCB) - (fIX, Seq_9) - (3'AAV ITR)
(5'AAV ITR) - (5'HSh-HCB) - (fIX, Seq_10) - (3'AAV ITR)

(5'AAV ITR) - (3'HSh-HCB) - (transgene) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (fVIII) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (fVIII-B-domain deleted) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (ET3) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (ET3, Seq_12) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (ET3, Seq_11) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (HSQ) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (HSQ, Seq_2) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (fIX) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (fIX, Seq_124) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (fIX, Seq_8) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (fIX, Seq_9) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (fIX, Seq_10) - (3'AAV ITR)

(5'AAV ITR) - (ABP-HP1-God-TSS) - (transgene) - (3'AAV ITR)
(5'AAV ITR) - (ABP-HP1-God-TSS) - (fVIII) - (3'AAV ITR)
(5'AAV ITR) - (ABP-HP1-God-TSS) - (fVIII-B-domain deleted) - (3'AAV ITR)
(5'AAV ITR) - (ABP-HP1-God-TSS) - (ET3) - (3'AAV ITR)
(5'AAV ITR) - (ABP-HP1-God-TSS) - (ET3, Seq_12) - (3'AAV ITR)
(5'AAV ITR) - (ABP-HP1-God-TSS) - (ET3, Seq_11) - (3'AAV ITR)
(5'AAV ITR) - (ABP-HP1-God-TSS) - (HSQ) - (3'AAV ITR)

(5'AAV ITR) - (ABP-HP1-God-TSS) - (HSQ, Seq_2) - (3'AAV ITR)
(5'AAV ITR) - (ABP-HP1-God-TSS) - (fIX) - (3'AAV ITR)
(5'AAV ITR) - (ABP-HP1-God-TSS) - (fIX, Seq_124) - (3'AAV ITR)
(5'AAV ITR) - (ABP-HP1-God-TSS) - (fIX, Seq_8) - (3'AAV ITR)
(5'AAV ITR) - (ABP-HP1-God-TSS) - (fIX, Seq_9) - (3'AAV ITR)
(5'AAV ITR) - (ABP-HP1-God-TSS) - (fIX, Seq_10) - (3'AAV ITR)

(5'AAV ITR) - (HSh-SynO-TSS) - (transgene) - (3'AAV ITR)
(5'AAV ITR) - (HSh-SynO-TSS) - (fVIII) - (3'AAV ITR)
(5'AAV ITR) - (HSh-SynO-TSS) - (fVIII-B-domain deleted) - (3'AAV ITR)
(5'AAV ITR) - (HSh-SynO-TSS) - (ET3) - (3'AAV ITR)
(5'AAV ITR) - (HSh-SynO-TSS) - (ET3, Seq_12) - (3'AAV ITR)
(5'AAV ITR) - (HSh-SynO-TSS) - (ET3, Seq_11) - (3'AAV ITR)
(5'AAV ITR) - (HSh-SynO-TSS) - (HSQ) - (3'AAV ITR)
(5'AAV ITR) - (HSh-SynO-TSS) - (HSQ, Seq_2) - (3'AAV ITR)
(5'AAV ITR) - (HSh-SynO-TSS) - (fIX) - (3'AAV ITR)
(5'AAV ITR) - (HSh-SynO-TSS) - (fIX, Seq_124) - (3'AAV ITR)
(5'AAV ITR) - (HSh-SynO-TSS) - (fIX, Seq_8) - (3'AAV ITR)
(5'AAV ITR) - (HSh-SynO-TSS) - (fIX, Seq_9) - (3'AAV ITR)
(5'AAV ITR) - (HSh-SynO-TSS) - (fIX, Seq_10) - (3'AAV ITR)

(5'AAV ITR) - (sHS-SynO-TSS) - (transgene) - (3'AAV ITR)
(5'AAV ITR) - (sHS-SynO-TSS) - (fVIII) - (3'AAV ITR)
(5'AAV ITR) - (sHS -SynO-TSS) - (fVIII-B-domain deleted) - (3'AAV ITR)
(5'AAV ITR) - (sHS -SynO-TSS) - (ET3) - (3'AAV ITR)
(5'AAV ITR) - (sHS -SynO-TSS) - (ET3, Seq_12) - (3'AAV ITR)
(5'AAV ITR) - (sHS -SynO-TSS) - (ET3, Seq_11) - (3'AAV ITR)
(5'AAV ITR) - (sHS -SynO-TSS) - (HSQ) - (3'AAV ITR)
(5'AAV ITR) - (sHS -SynO-TSS) - (HSQ, Seq_2) - (3'AAV ITR)
(5'AAV ITR) - (sHS -SynO-TSS) - (fIX) - (3'AAV ITR)
(5'AAV ITR) - (sHS -SynO-TSS) - (fIX, Seq_124) - (3'AAV ITR)
(5'AAV ITR) - (sHS -SynO-TSS) - (fIX, Seq_8) - (3'AAV ITR)
(5'AAV ITR) - (sHS -SynO-TSS) - (fIX, Seq_9) - (3'AAV ITR)
(5'AAV ITR) - (sHS -SynO-TSS) - (fIX, Seq_10) - (3'AAV ITR)

The transgene can be flanked by regulatory sequences such as a 5' Kozak sequence and/or a 3' polyadenylation signal. For example, in some embodiments, the recombinant AAV vector can have a genome with a structure set forth as one of:

(5'AAV ITR) - (HCB) - (Kozak) - (transgene) - (polyA signal) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HCB) - (Kozak) - (fVIII) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HCB) - (Kozak) - (fVIII-B-domain deleted) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HCB) - (Kozak) - (ET3) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HCB) - (Kozak) - (ET3, Seq_12) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HCB) - (Kozak) - (ET3, Seq_11) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HCB) - (Kozak) - (HSQ) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HCB) - (Kozak) - (HSQ, Seq_2) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HCB) - (Kozak) - (fIX) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HCB) - (Kozak) - (fIX, Seq_124) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HCB) - (Kozak) - (fIX, Seq_8) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HCB) - (Kozak) - (fIX, Seq_9) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HCB) - (Kozak) - (fIX, Seq_10) - (polyA signal) - (3'AAV ITR)

(5'AAV ITR) - (HSh-HCB) - (Kozak) - (transgene) - (polyA signal) - (3'AAV ITR)

(5'AAV ITR) - (HSh-HCB) - (Kozak) - (fVIII) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HSh-HCB) - (Kozak) - (fVIII-B-domain deleted) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HSh-HCB) - (Kozak) - (ET3) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HSh-HCB) - (Kozak) - (ET3, Seq_12) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HSh-HCB) - (Kozak) - (ET3, Seq_11) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HSh-HCB) - (Kozak) - (HSQ) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HSh-HCB) - (Kozak) - (HSQ, Seq_2) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HSh-HCB) - (Kozak) - (fIX) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HSh-HCB) - (Kozak) - (fIX, Seq_124) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HSh-HCB) - (Kozak) - (fIX, Seq_8) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HSh-HCB) - (Kozak) - (fIX, Seq_9) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HSh-HCB) - (Kozak) - (fIX, Seq_10) - (polyA signal) - (3'AAV ITR)

(5'AAV ITR) - (5'HSh-HCB) - (Kozak) - (transgene) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (5'HSh-HCB) - (Kozak) - (fVIII) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (5'HSh-HCB) - (Kozak) - (fVIII-B-domain deleted) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (5'HSh-HCB) - (Kozak) - (ET3) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (5'HSh-HCB) - (Kozak) - (ET3, Seq_12) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (5'HSh-HCB) - (Kozak) - (ET3, Seq_11) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (5'HSh-HCB) - (Kozak) - (HSQ) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (5'HSh-HCB) - (Kozak) - (HSQ, Seq_2) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (5'HSh-HCB) - (Kozak) - (fIX) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (5'HSh-HCB) - (Kozak) - (fIX, Seq_124) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (5'HSh-HCB) - (Kozak) - (fIX, Seq_8) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (5'HSh-HCB) - (Kozak) - (fIX, Seq_9) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (5'HSh-HCB) - (Kozak) - (fIX, Seq_10) - (polyA signal) - (3'AAV ITR)

(5'AAV ITR) - (3'HSh-HCB) - (Kozak) - (transgene) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (Kozak) - (fVIII) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (Kozak) - (fVIII-B-domain deleted) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (Kozak) - (ET3) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (Kozak) - (ET3, Seq_12) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (Kozak) - (ET3, Seq_11) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (Kozak) - (HSQ) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (Kozak) - (HSQ, Seq_2) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (Kozak) - (fIX) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (Kozak) - (fIX, Seq_124) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (Kozak) - (fIX, Seq_8) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (Kozak) - (fIX, Seq_9) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (3'HSh-HCB) - (Kozak) - (fIX, Seq_10) - (polyA signal) - (3'AAV ITR)

(5'AAV ITR) - (ABP-HP1-God-TSS) - (Kozak) - (transgene) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (ABP-HP1-God-TSS) - (Kozak) - (fVIII) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (ABP-HP1-God-TSS) - (Kozak) - (fVIII-B-domain deleted) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (ABP-HP1-God-TSS) - (Kozak) - (ET3) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (ABP-HP1-God-TSS) - (Kozak) - (ET3, Seq_12) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (ABP-HP1-God-TSS) - (Kozak) - (ET3, Seq_11) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (ABP-HP1-God-TSS) - (Kozak) - (HSQ) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (ABP-HP1-God-TSS) - (Kozak) - (HSQ, Seq_2) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (ABP-HP1-God-TSS) - (Kozak) - (fIX) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (ABP-HP1-God-TSS) - (Kozak) - (fIX, Seq_124) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (ABP-HP1-God-TSS) - (Kozak) - (fIX, Seq_8) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (ABP-HP1-God-TSS) - (Kozak) - (fIX, Seq_9) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (ABP-HP1-God-TSS) - (Kozak) - (fIX, Seq_10) - (polyA signal) - (3'AAV ITR)

(5'AAV ITR) - (HSh-SynO-TSS) - (Kozak) - (transgene) - (polyA signal) - (3'AAV ITR)

(5'AAV ITR) - (HSh-SynO-TSS) - (Kozak) - (fVIII) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HSh-SynO-TSS) - (Kozak) - (fVIII-B-domain deleted) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HSh-SynO-TSS) - (Kozak) - (ET3) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HSh-SynO-TSS) - (Kozak) - (ET3, Seq_12) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HSh-SynO-TSS) - (Kozak) - (ET3, Seq_11) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HSh-SynO-TSS) - (Kozak) - (HSQ) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HSh-SynO-TSS) - (Kozak) - (HSQ, Seq_2) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HSh-SynO-TSS) - (Kozak) - (fIX) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HSh-SynO-TSS) - (Kozak) - (fIX, Seq_124) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HSh-SynO-TSS) - (Kozak) - (fIX, Seq_8) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HSh-SynO-TSS) - (Kozak) - (fIX, Seq_9) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (HSh-SynO-TSS) - (Kozak) - (fIX, Seq_10) - (polyA signal) - (3'AAV ITR)

(5'AAV ITR) - (sHS-SynO-TSS) - (Kozak) - (transgene) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (sHS-SynO-TSS) - (Kozak) - (fVIII) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (sHS -SynO-TSS) - (Kozak) - (fVIII-B-domain deleted) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (sHS -SynO-TSS) - (Kozak) - (ET3) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (sHS -SynO-TSS) - (Kozak) - (ET3, Seq_12) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (sHS -SynO-TSS) - (Kozak) - (ET3, Seq_11) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (sHS -SynO-TSS) - (Kozak) - (HSQ) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (sHS -SynO-TSS) - (Kozak) - (HSQ, Seq_2) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (sHS -SynO-TSS) - (Kozak) - (fIX) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (sHS -SynO-TSS) - (Kozak) - (fIX, Seq_124) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (sHS -SynO-TSS) - (Kozak) - (fIX, Seq_8) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (sHS -SynO-TSS) - (Kozak) - (fIX, Seq_9) - (polyA signal) - (3'AAV ITR)
(5'AAV ITR) - (sHS -SynO-TSS) - (Kozak) - (fIX, Seq_10) - (polyA signal) - (3'AAV ITR)

The AAV ITRs, and other selected AAV components described herein, may be readily selected from among any AAV serotype, including, without limitation, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9 and function variants thereof. These ITRs or other AAV components may be readily isolated using techniques available to those of skill in the art from an AAV serotype. Such AAV may be isolated or obtained from academic, commercial, or public sources (*e.g.*, the American Type Culture Collection, Manassas, Va.). Alternatively, the AAV sequences may be obtained through synthetic or other suitable means by reference to published sequences such as are available in the literature or in databases such as, *e.g.*, GenBank, PubMed, or the like.

In some embodiments, the vector can be a recombinant AAV vector comprising a genome comprising a nucleic acid molecule encoding any of the liver-specific promoters provided herein (such as the HCB promoter, SEQ ID NO: 4) operably linked to a heterologous nucleic molecule encoding a fVIII variant, wherein the heterologous nucleic acid molecule comprises or consists of the nucleic acid sequence set forth as SEQ ID NO: 2, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 125, or SEQ ID NO: 126, or a nucleic acid sequence at least 90% identical to SEQ ID NO: 2, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 125, or SEQ ID NO: 126. Is several such embodiments, the recombinant AAV genome (from 5' to 3' ITR) is no more than 5.1, 5.0, 4.9, 4.8, 4.7, 4.6, or 4.5 kb in length.

In some embodiments, the vector can be a recombinant AAV vector comprising a genome comprising a nucleic acid molecule encoding any of the liver-specific promoters provided herein (such as the HCB promoter, SEQ ID NO: 4) operably linked to a heterologous nucleic molecule encoding a fIX variant, wherein the heterologous nucleic acid molecule comprises or consists of the nucleic acid

sequence set forth as SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 124, or SEQ ID NO: 127, or a nucleic acid sequence at least 90% identical SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 124, or SEQ ID NO: 127. Is several such embodiments, the recombinant AAV genome (from 5' to 3' ITR) is no more than 5.1, 5.0, 4.9, 4.8, 4.7, 4.6, or 4.5 kb in length.

AAV is currently one of the most frequently used viruses for gene therapy. Although AAV infects humans and some other primate species, it is not known to cause disease and elicits a very mild immune response. Gene therapy vectors that utilize AAV can infect both dividing and quiescent cells and persist in an extrachromosomal state without integrating into the genome of the host cell. Because of the advantageous features of AAV, the present disclosure contemplates the use of AAV for the recombinant nucleic acid molecules and methods disclosed herein.

AAV possesses several desirable features for a gene therapy vector, including the ability to bind and enter target cells, enter the nucleus, the ability to be expressed in the nucleus for a prolonged period of time, and low toxicity. However, the small size of the AAV genome limits the size of heterologous DNA that can be incorporated. To minimize this problem, AAV vectors have been constructed that do not encode Rep and the integration efficiency element (IEE). The ITRs are retained as they are *cis* signals required for packaging (Daya and Berns, *Clin Microbiol Rev* 21(4):583-593, 2008).

Methods for producing rAAV suitable for gene therapy are known (see, for example, U.S. Patent Application Nos. 2012/0100606; 2012/0135515; 2011/0229971; and 2013/0072548; and Ghosh *et al.*, *Gene Ther* 13(4):321-329, 2006), and can be utilized with the recombinant nucleic acid molecules and methods disclosed herein.

In some embodiments, the nucleic acids disclosed herein are part of an expression cassette or transgene. See *e.g.*, US Pat. App. Pub. 20150139953. The expression cassette is composed of a transgene and regulatory sequences, *e.g.*, promotor and 5' and 3' AAV inverted terminal repeats (ITRs). In one desirable embodiment, the ITRs of AAV serotype 2 or 8 are used. However, ITRs from other suitable serotypes may be selected. An expression cassette is typically packaged into a capsid protein and delivered to a selected host cell.

In some embodiments, the disclosure provides for a method of generating a recombinant adeno-associated virus (AAV) having an AAV serotype capsid, or a portion thereof. Such a method involves culturing a host cell which contains a nucleic acid sequence encoding an adeno-associated virus (AAV) serotype capsid protein; a functional rep gene; an expression cassette composed of AAV inverted terminal repeats (ITRs) and a transgene; and sufficient helper functions to permit packaging of the expression cassette into the AAV capsid protein. See *e.g.*, US Pat. App. Pub. 20150139953.

The components for culturing in the host cell to package an AAV expression cassette in an AAV capsid may be provided to the host cell in trans. Alternatively, any one or more of the components (*e.g.*, expression cassette, rep sequences, cap sequences, and/or helper functions) may be provided by a stable host cell which has been engineered to contain one or more of the required components using methods known to those of skill in the art. Most suitably, such a stable host cell will contain the component(s) under the control of an inducible promoter. However, the required component(s) may be under the control of a constitutive promoter. In still another alternative, a selected stable host cell may contain selected

component(s) under the control of a constitutive promoter and other selected component(s) under the control of one or more inducible promoters. For example, a stable host cell may be generated which is derived from 293 cells (which contain E1 helper functions under the control of a constitutive promoter), but which contains the rep and/or cap proteins under the control of inducible promoters. Still other stable host cells may be generated by one of skill in the art.

In some embodiments, the disclosure relates to recombinant vectors comprising a liver specific promotor nucleic acid sequence in operable combination with transgene. The transgene is a nucleic acid sequence, heterologous to the vector sequences flanking the transgene, which encodes a protein, or other product, of interest. The nucleic acid coding sequence is operatively linked to regulatory components in a manner which permits transgene transcription, translation, and/or expression in a host cell.

A typical transgene is a sequence encoding a product which is useful in biology and medicine, such as proteins, peptides, RNA, enzymes, dominant negative mutants, or catalytic RNAs. Desirable RNA molecules include mRNA, tRNA, dsRNA, ribosomal RNA, catalytic RNAs, siRNA, microRNA, small hairpin RNA, trans-splicing RNA, and antisense RNAs. One example of a useful RNA sequence is a sequence which inhibits or extinguishes expression of a targeted nucleic acid sequence in the treated animal. Typically, suitable target sequences in include oncologic targets and viral diseases.

The transgene may be used to correct or ameliorate gene deficiencies, which may include deficiencies in which normal genes are expressed at less than normal levels or deficiencies in which the functional gene product is not expressed. A preferred type of transgene sequence encodes a therapeutic protein or polypeptide which is expressed in a host cell. The disclosure further contemplates using multiple transgenes, *e.g.*, to correct or ameliorate a gene defect caused by a multi-subunit protein. In certain situations, a different transgene may be used to encode each subunit of a protein, or to encode different peptides or proteins. This is desirable when the size of the DNA encoding the protein subunit is large, *e.g.*, for an immunoglobulin, the platelet-derived growth factor, or a dystrophin protein. In order for the cell to produce the multi-subunit protein, a cell is infected with the recombinant virus containing each of the different subunits. Alternatively, different subunits of a protein may be encoded by the same transgene. In this case, a single transgene includes the DNA encoding each of the subunits, with the DNA for each subunit separated by an internal ribozyme entry site (IRES). This is desirable when the size of the DNA encoding each of the subunits and cis-regulatory control regions such as a promoter, intron, polyA signal is small, *e.g.*, the total size of the DNA encoding the subunits and the IRES and cis-regulatory control regions is less than five kilobases. As an alternative to an IRES, the DNA may be separated by sequences encoding a 2A peptide, which self-cleaves in a post-translational event. See, *e.g.*, M. L. Donnelly, et al., J. Gen. Virol., 78(Pt 1):13-21 (Jan 1997); Furler, S., et al, Gene Ther., 8(11):864-873 (June 2001); Klump H., et al., Gene Ther., 8(10):811-817 (May 2001). In certain embodiments, rAAV carrying the desired transgene(s) or subunits are co-administered to allow them to concatamerize *in vivo* to form a single vector genome. In such an embodiment, a first AAV may carry an expression cassette which expresses a single transgene and a second AAV may carry an expression cassette which expresses a different transgene for co-expression in the host cell. However, the selected transgene may encode any biologically active product or other product, *e.g.*, a product desirable for study.

The expression cassette can be carried on any suitable vector, *e.g.*, a plasmid, which is delivered to a host cell. The plasmids useful in this disclosure may be engineered such that they are suitable for replication and, optionally, integration in prokaryotic cells, mammalian cells, or both. These plasmids (or other vectors carrying the 5' AAV ITR-heterologous molecule-3' ITR) contain sequences permitting replication of the expression cassette in eukaryotes and/or prokaryotes and selection markers for these systems. Preferably, the molecule carrying the expression cassette is transfected into the cell, where it may exist transiently. Alternatively, the expression cassette (carrying the 5' AAV ITR-heterologous molecule-3' ITR) may be stably integrated into the genome of the host cell, either chromosomally or as an episome. In certain embodiments, the expression cassette may be present in multiple copies, optionally in head-to-head, head-to-tail, or tail-to-tail concatamers. Suitable transfection techniques are known and may readily be utilized to deliver the expression cassette to the host cell.

Generally, when delivering the vector comprising the expression cassette by transfection, the vector and the relative amounts of vector DNA to host cells may be adjusted, taking into consideration such factors as the selected vector, the delivery method and the host cells selected. In addition to the expression cassette, the host cell contains the sequences which drive expression of the AAV capsid protein in the host cell and rep sequences of the same serotype as the serotype of the AAV ITRs found in the expression cassette, or a cross-complementing serotype. Although the molecule(s) providing rep and cap may exist in the host cell transiently (*i.e.*, through transfection), it is preferred that one or both of the rep and cap proteins and the promoter(s) controlling their expression be stably expressed in the host cell, *e.g.*, as an episome or by integration into the chromosome of the host cell.

The packaging host cell also typically contains helper functions in order to package the rAAV of the disclosure. Optionally, these functions may be supplied by a herpesvirus. Most desirably, the necessary helper functions are each provided from a human or non-human primate adenovirus source, such as those described above and/or are available from a variety of sources, including the American Type Culture Collection (ATCC), Manassas, Va. (US). The desired helper functions, can be provided using any means that allows their expression in a cell.

Introduction into the host cell of the vector may be achieved by any means known in the art or as disclosed above, including transfection, infection, electroporation, liposome delivery, membrane fusion techniques, high velocity DNA-coated pellets, viral infection and protoplast fusion, among others. One or more of the adenoviral genes may be stably integrated into the genome of the host cell, stably expressed as episomes, or expressed transiently. The gene products may all be expressed transiently, on an episome or stably integrated, or some of the gene products may be expressed stably while others are expressed transiently. Furthermore, the promoters for each of the adenoviral genes may be selected independently from a constitutive promoter, an inducible promoter or a native adenoviral promoter. The promoters may be regulated by a specific physiological state of the organism or cell (*i.e.*, by the differentiation state or in replicating or quiescent cells) or by exogenously added factors, for example.

Introduction of the molecules (as plasmids or viruses) into the host cell may be accomplished using techniques known to the skilled artisan. In preferred embodiment, standard transfection techniques are used, *e.g.*, $CaPO_4$ transfection or electroporation, and/or infection by hybrid adenovirus/AAV vectors

into cell lines such as the human embryonic kidney cell line HEK 293 (a human kidney cell line containing functional adenovirus E1 genes which provides trans-acting E1 proteins).

One of skill in the art will readily understand that the AAV techniques can be adapted for use in these and other viral vector systems for *in vitro*, ex vivo or *in vivo* gene delivery. The in certain embodiments the disclosure contemplates the use of nucleic acids and vectors disclosed herein in a variety of rAAV and non-rAAV vector systems. Such vectors systems may include, *e.g.*, lentiviruses, retroviruses, poxviruses, vaccinia viruses, and adenoviral systems, among others.

In some embodiments, it is contemplated that viral particles, nucleic acids and vectors disclosed herein are useful for a variety of purposes, including for delivery of therapeutic molecules for gene expression of therapeutic proteins.

Therapeutic proteins encoded by the nucleic acids (*e.g.*, operably in combination with promoters) reported herein include those used for treatment of hemophilia, including hemophilia B (including fIX) and hemophilia A (including fVIII and its variants, such as the light chain and heavy chain of the heterodimer and the B-deleted domain; U.S. Pat. No. 6,200,560 and U.S. Pat. No. 6,221,349). The Factor VIII gene codes for 2351 amino acids and the protein has six domains, designated from the amino to the terminal carboxy terminus as A1-A2-B-A3-C1-C2 [Wood et al, Nature, 312:330 (1984); Vehar et al., Nature 312:337 (1984); and Toole et al, Nature, 342:337 (1984)]. Human fVIII is processed within the cell to yield a heterodimer primarily comprising a heavy chain containing the A1, A2 and B domains and a light chain containing the A3, C1 and C2 domains. Both the single chain polypeptide and the heterodimer circulate in the plasma as inactive precursors, until activated by thrombin cleavage between the A2 and B domains, which releases the B domain and results in a heavy chain consisting of the A1 and A2 domains. The B domain is deleted in the activated procoagulant form of the protein. Additionally, in the native protein, two polypeptide chains ("a" and "b"), flanking the B domain, are bound to a divalent calcium cation.

A treatment option for a patient diagnosed with hemophilia A is the exogenous administration of recombinant fVIII sometimes referred to as fVIII replacement therapy. In some patients, this therapy can lead to the development of antibodies that bind to the administered fVIII protein. Subsequently, the fVIII-antibody bound conjugates, typically referred to as inhibitors, interfere with or retard the ability of fVIII to cause blood clotting. Inhibitory autoantibodies also sometimes occur spontaneously in a subject that is not genetically at risk of having hemophilia, termed acquired hemophilia. Inhibitory antibodies assays are typically performed prior to exogenous fVIII treatment in order to determine whether the anti-coagulant therapy will be effective.

A "Bethesda assay" has historically been used to quantitate the inhibitory strength the concentration of fVIII binding antibodies. In the assay, serial dilutions of plasma from a patient, *e.g.*, prior to having surgery, are prepared and each dilution is mixed with an equal volume of normal plasma as a source of fVIII. After incubating for a couple hours, the activities of fVIII in each of the diluted mixtures are measured. Having antibody inhibitor concentrations that prevent fVIII clotting activity after multiple repeated dilutions indicates a heightened risk of uncontrolled bleeding. Patients with inhibitor titers after about ten dilutions are felt to be unlikely to respond to exogenous fVIII infusions to stop

bleeding. A Bethesda titer is defined as the reciprocal of the dilution that results in 50% inhibition of FVIII activity present in normal human plasma. A Bethesda titer greater than 10 is considered the threshold of response to FVIII replacement therapy.

In certain embodiments, the disclosure relates to methods of inducing blood clotting comprising administering an effective amount of a viral particle or capsid comprising a vector comprising a nucleic acid encoding a blood clotting factor as disclosed herein to a subject in need thereof.

In certain embodiments, the subject is diagnosed with hemophilia A or B or acquired hemophilia or unlikely to respond to exogenous fVIII infusions.

In some embodiments, this disclosure relates to methods gene transfer for the treatment of hemophilia B using an adeno-associated viral (AAV) vector encoding human fIX as the gene delivery vehicle. While several such AAV-based gene therapies for hemophilia B have entered into human clinical trials, they have been hampered by low expression of the therapeutic protein, clotting fIX, after administration of the virus resulting on only partial correction of the disease. AAV vector toxicity limits the dose of the virus that may be safely administered. To successfully transition to a clinically viable therapy, a vector should provide efficacious expression of fIX at viral doses below the threshold of toxicity.

Treating patients with inhibitors to fVIII has also been accomplished by methods of immune tolerance induction (ITI) which typically involves the daily infusion of fVIII until circulating inhibitor/antibody levels decline. However, 20-30% of patients fail to become tolerant after an immune tolerance induction (ITI) therapy. Persistence of fVIII inhibitors is associated with increased risks of morbidity and mortality. In certain embodiments, the disclosure relates to methods of immune tolerance induction comprising administering an effective amount of a vector or a capsid as disclosed herein to a subject in need thereof.

In some embodiments, the therapeutic proteins encoded by the nucleic acids (*e.g.*, operably in combination with promoters) reported herein comprises first 57 base pairs of the fVIII heavy chain which encodes the 10 amino acid signal sequence, as well as the human beta globin polyadenylation sequence or growth hormone (hGH) polyadenylation sequence. In alternative embodiments, the A1 and A2 domains, as well as 5 amino acids from the N-terminus of the B domain, and/or 85 amino acids of the C-terminus of the B domain, as well as the A3, C1 and C2 domains. In yet other embodiments, the nucleic acids encoding fVIII heavy chain and light chain are provided in a single nucleic acid separated by 42 nucleic acids coding for 14 amino acids of the B domain. See U.S. Pat. No. 6,200,560.

As used herein, a therapeutically effective amount is an amount of AAV vector that produces sufficient amounts of fVIII to decrease the time it takes for the blood of a subject to clot. Generally, severe hemophiliacs having less than 1% of normal levels of fVIII have a whole blood clotting time of greater than 60 minutes as compared to approximately 10 minutes for non-hemophiliacs.

The present disclosure is not limited to any specific fVIII or fIX or other protein sequence reported herein. Many natural and recombinant forms of fVIII have been isolated and generated. Examples of naturally occurring and recombinant forms of fVII can be found in the patent and scientific literature including, U.S. Pat. No. 5,563,045, U.S. Pat. No. 5,451,521, U.S. Pat. No. 5,422,260, U.S. Pat.

No. 5,004,803, U.S. Pat. No. 4,757,006, U.S. Pat. No. 5,661,008, U.S. Pat. No. 5,789,203, U.S. Pat. No. 5,681,746, U.S. Pat. No. 5,595,886, U.S. Pat. No. 5,045,455, U.S. Pat. No. 5,668,108, U.S. Pat. No. 5,633,150, U.S. Pat. No. 5,693,499, U.S. Pat. No. 5,587,310, U.S. Pat. No. 5,171,844, U.S. Pat. No. 5,149,637, U.S. Pat. No. 5,112,950, U.S. Pat. No. 4,886,876, WO 94/11503, WO 87/07144, WO 92/16557, WO 91/09122, WO 97/03195, WO 96/21035, WO 91/07490, EP 0 672 138, EP 0 270 618, EP 0 182 448, EP 0 162 067, EP 0 786 474, EP 0 533 862, EP 0 506 757, EP 0 874 057, EP 0 795 021, EP 0 670 332, EP 0 500 734, EP 0 232 112, EP 0 160 457, Sanberg et al., Int. Congress of the World Fed. Of Hemophilia (1992), and Lind et al., Eur. J. Biochem., 232:19 (1995).

Furthermore, the disclosure is not limited to human fVIII. Indeed, it is intended that the present disclosure encompass fVIII from animals other than humans, including but not limited to companion animals (*e.g.*, canine, felines, and equines), livestock (*e.g.*, bovines, caprines and ovines), laboratory animals, marine mammals, large cats, etc.

The AAV vectors may contain a nucleic acid coding for fragments of fVIII which is itself not biologically active, yet when administered into the subject improves or restores the blood clotting time. For example, the fVIII protein comprises two polypeptide chains: a heavy chain and a light chain separated by a B-domain which is cleaved during processing. Co-transducing recipient cells with the FVIII heavy and light chains leads to the expression of biologically active fVIII. Administration of only the chain defective is contemplated in patients because most hemophiliacs contain a mutation or deletion in only one of the chains (*e.g.*, heavy or light chain).

Thus, in certain embodiments, the disclosure relates to vectors disclosed herein having nucleic acids encoding a light chain containing the A3, C1 and C2 domains or a heavy chain consisting of the A1 and A2 domains.

Additional description of recombinant vectors and therapeutic modalities

The recombinant vectors disclosed herein (for example, a recombinant AAV vector) can be used in several different therapeutic applications, depending on the protein of interest encoded by the recombinant vector.

In certain embodiments, the uses are for the treatment of hereditary hemochromatosis (HH), a major disorder of iron overload, Wilson's disease, a genetic disorder of copper overload, and alpha1-antitrypsin (α1-AT) deficiency. In certain embodiments, the protein is human Alpha1-antitrypsin (α1-AT, Accession: P01009.3), HFE protein (Accession NP_000401.1 or Q30201), or hepatic protein ATP7B (Accession P35670.4) or variants with greater than 50, 60, 70, 80, 90, 95, or 95 sequence identity or similarity.

In certain embodiments, the use is for the treatment of hypercholesterolaemia using a promotor herein in operable combination with a nucleic acid that encodes for human phenylalanine hydroxylase (Accession: P00439.1) or variants with greater than 50, 60, 70, 80, 90, 95, or 95 sequence identity or similarity.

In certain embodiments, the use is for the treatment of Type 1 tyrosinemia using a promotor herein in operable combination with a nucleic acid that encodes for human fumarylacetoacetate hydrolase

(Accession: P16930.2) or variants with greater than 50, 60, 70, 80, 90, 95, or 95 sequence identity or similarity.

In certain embodiments, the use is for the treatment of Type 2 tyrosinemia using a promotor herein in operable combination with a nucleic acid that encodes for human tyrosine aminotransferase (Accession: P17735.1) or variants with greater than 50, 60, 70, 80, 90, 95, or 95 sequence identity or similarity.

In certain embodiments, the use is for the treatment of homocystinuria and hyperhomocysteinemia using a promotor herein in operable combination with a nucleic acid that encodes for human methylenetetrahydrofolate reductase (Accession: P42898.3) or variants with greater than 50, 60, 70, 80, 90, 95, or 95 sequence identity or similarity.

In certain embodiments, the use is for the treatment of hyperlipidemia and hypercholesterolemia using a promotor herein in operable combination with a nucleic acid that encodes for human medium chain acyl-CoA dehydrogenase (Accession: P11310.1) or variants with greater than 50, 60, 70, 80, 90, 95, or 95 sequence identity or similarity.

In certain embodiments, the use is for the treatment of Galactosemia using a promotor herein in operable combination with a nucleic acid that encodes for human galactose-1-phosphate uridyl transferase (Accession: P07902.3) or variants with greater than 50, 60, 70, 80, 90, 95, or 95 sequence identity or similarity.

In certain embodiments, the use is for the treatment of Lesch-Nyhan syndrome using a promotor herein in operable combination with a nucleic acid that encodes for human hypoxanthine phosphoribosyl-transferase (Accession: P00492.2) or variants with greater than 50, 60, 70, 80, 90, 95, or 95 sequence identity or similarity.

In certain embodiments, the use is for the treatment of Gaucher disease using a promotor herein in operable combination with a nucleic acid that encodes for human cerebrosidase (Accession: P07602.2, Accession: P04062.3) or variants with greater than 50, 60, 70, 80, 90, 95, or 95 sequence identity or similarity.

In certain embodiments, the use is for the treatment of Tay-Sachs disease using a promotor herein in operable combination with a nucleic acid that encodes for human beta-hexosaminidase A (Accession: P06865.2) or variants with greater than 50, 60, 70, 80, 90, 95, or 95 sequence identity or similarity.

In certain embodiments, the use is for the treatment of Fabry disease using a promotor in operable combination with a nucleic acid that encodes for human α-galactosidase (Accession: P06280.1) or variants with greater than 50, 60, 70, 80, 90, 95, or 95 sequence identity or similarity.

In certain embodiments, the use is for the treatment of Hunter syndrome using a promotor in operable combination with a nucleic acid that encodes for human iduronate sulphatase (Accession: P22304.1) or variants with greater than 50, 60, 70, 80, 90, 95, or 95 sequence identity or similarity.

In certain embodiments, the use is for the treatment of glycogen storage disease type Ia using a promotor in operable combination with a nucleic acid that encodes for human glucose-6-phosphatase (Accession: P35575.2) or variants with greater than 50, 60, 70, 80, 90, 95, or 95 sequence identity or similarity.

In certain embodiments, the use is for the treatment of ammonia metabolism using a promotor in operable combination with a nucleic acid that encodes for human ornithine transcarbamylase (Accession: P00480.3) or variants with greater than 50, 60, 70, 80, 90, 95, or 95 sequence identity or similarity.

In certain embodiments, the use is for the treatment of phenylketonuria using a promotor in operable combination with a nucleic acid that encodes for human low-density lipoprotein receptor (Accession: P01130.1) or variants with greater than 50, 60, 70, 80, 90, 95, or 95 sequence identity or similarity.

In certain embodiments, the use is for the treatment of propionic acidemia using a promotor in operable combination with a nucleic acid that encodes for human propionyl-coenzyme A carboxylase, either PCCA and/or PCCB (Accession: P05166.3 beta, NP_000273.2 alpha , NP_001121164.1 alpha) or variants with greater than 50, 60, 70, 80, 90, 95, or 95 sequence identity or similarity.

Also contemplated are uses in vaccine regimens, *e.g.*, for co-delivery of a cytokine, or for delivery of an immunogen or antigen.

Recombinant virus particles, capsids, or vectors comprising nucleic acids disclosed herein can be delivered to liver via the hepatic artery, the portal vein, or intravenously to yield therapeutic levels of therapeutic proteins or clotting factors in the blood. The capsid or vector is preferably suspended in a physiologically compatible carrier, may be administered to a human or non-human mammalian patient. Suitable carriers may be readily selected by one of skill in the art in view of the indication for which the transfer virus is directed. For example, one suitable carrier includes saline, which may be formulated with a variety of buffering solutions (*e.g.*, phosphate buffered saline). Other exemplary carriers include sterile saline, lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, sesame oil, and water.

Optionally, the compositions of the disclosure may contain other pharmaceutically acceptable excipients, such as preservatives, or chemical stabilizers. Suitable exemplary preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and parachlorophenol. Suitable chemical stabilizers include gelatin and albumin.

The recombinant virus particles, capsids, or vectors are administered in sufficient amounts to transfect the cells and to provide sufficient levels of gene transfer and expression to provide a therapeutic benefit without undue adverse effects, or with medically acceptable physiological effects, which can be determined by those skilled in the medical arts. Conventional and pharmaceutically acceptable routes of administration include, but are not limited to, direct delivery to a desired organ (*e.g.*, the liver (optionally via the hepatic artery) or lung), oral, inhalation, intranasal, intratracheal, intraarterial, intraocular, intravenous, intramuscular, subcutaneous, intradermal, and other parental routes of administration. Routes of administration may be combined, if desired.

Dosages of the recombinant virus particles, capsids, or vectors will depend primarily on factors such as the condition being treated, the age, weight and health of the patient, and may thus vary among patients. For example, a therapeutically effective human dosage of the viral vector is generally in the range of from about 0.1 ml to about 100 ml of solution containing concentrations of from about $1x10^9$ to $1x10^{16}$ genomes virus vector.

Other useful therapeutic proteins encoded by the nucleic acids (*e.g.*, operably in combination with promoters) reported herein include hormones and growth and differentiation factors including, without limitation, 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), platelet-derived growth factor (PDGF), insulin growth factors I and II (IGF-I and IGF-II), any one of the transforming growth factor alpha superfamily, including TGFalpha, activins, inhibins, or any of the bone morphogenic proteins (BMP) BMPs 1-15, any one of the heregluin/neuregulin/ ARIA/neu differentiation factor (NDF) family of growth factors, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophins NT-3 and NT-4/5, ciliary neurotrophic factor (CNTF), glial cell line derived neurotrophic factor (GDNF), neurturin, agrin, any one of the family of semaphorins/collapsins, netrin-1 and netrin-2, hepatocyte growth factor (HGF), ephrins, noggin, sonic hedgehog and tyrosine hydroxylase.

Other therapeutic proteins encoded by the nucleic acids (*e.g.*, operably in combination with promoters) reported herein include those that regulate the immune system including, without limitation, cytokines and lymphokines such as thrombopoietin (TPO), interleukins (IL) IL-1 through IL-25 (including IL-2, IL-4, IL-12 and IL-18), monocyte chemoattractant protein, leukemia inhibitory factor, granulocyte-macrophage colony stimulating factor, Fas ligand, tumor necrosis factors alpha and beta, interferons alpha, beta, and gamma, stem cell factor, flk-2/flt3 ligand. Proteins produced by the immune system are also useful. These include, without limitations, immunoglobulins 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, as well as engineered immunoglobulins and MHC molecules. Useful proteins also include complement regulatory proteins such as complement regulatory proteins, membrane cofactor protein (MCP), decay accelerating factor (DAF), CR1, CF2 and CD59.

Other therapeutic proteins encoded by the nucleic acids (*e.g.*, operably in combination with promoters) reported herein are receptors for the hormones, growth factors, cytokines, lymphokines, regulatory proteins and immune system proteins. The disclosure encompasses receptors for cholesterol regulation and/or lipid modulation, including the low density lipoprotein (LDL) receptor, high density lipoprotein (HDL) receptor, the very low density lipoprotein (VLDL) receptor, and scavenger receptors. The disclosure also encompasses proteins such as members of the steroid hormone receptor superfamily including glucocorticoid receptors and estrogen receptors, Vitamin D receptors and other nuclear receptors. In addition, useful proteins include transcription factors such as jun, fos, max, mad, serum response factor (SRF), AP-1, AP2, myb, MyoD and myogenin, ETS-box containing proteins, TFE3, E2F, ATF1, ATF2, ATF3, ATF4, ZF5, NFAT, CREB, HNF-4, C/EBP, SP1, CCAAT-box binding proteins, interferon regulation factor (IRF-1), Wilms tumor protein, ETS-binding protein, STAT, GATA-box binding proteins, *e.g.*, GATA-3, and the forkhead family of winged helix proteins.

Other useful proteins include, carbamoyl synthetase I, ornithine transcarbamylase, arginosuccinate synthetase, arginosuccinate lyase, arginase, fumarylacetacetate hydrolase, phenylalanine hydroxylase, alpha-1 antitrypsin, glucose-6-phosphatase, porphobilinogen deaminase, cystathione beta-synthase, branched chain ketoacid decarboxylase, albumin, isovaleryl-coA dehydrogenase, propionyl CoA carboxylase, methyl malonyl CoA mutase, glutaryl CoA dehydrogenase, insulin, beta-glucosidase, pyruvate carboxylate, hepatic phosphorylase, phosphorylase kinase, glycine decarboxylase, H-protein, T-protein, a cystic fibrosis transmembrane regulator (CFTR) sequence, and a dystrophin cDNA sequence. Still other useful proteins include enzymes such as may be useful in enzyme replacement therapy, which is useful in a variety of conditions resulting from deficient activity of enzyme. For example, enzymes that contain mannose-6-phosphate may be utilized in therapies for lysosomal storage diseases (*e.g.*, a suitable gene includes that encoding beta-glucuronidase (GUSB)).

Other useful proteins include non-naturally occurring polypeptides, such as chimeric or hybrid polypeptides having a non-naturally occurring amino acid sequence containing insertions, deletions or amino acid substitutions. For example, single-chain engineered immunoglobulins could be useful in certain immunocompromised patients. Other types of non-naturally occurring gene sequences include antisense molecules and catalytic nucleic acids, such as ribozymes, which could be used to reduce overexpression of a target.

Reduction and/or modulation of expression of a protein is particularly desirable for treatment of hyperproliferative conditions characterized by hyperproliferating cells, as are cancers and psoriasis. Target polypeptides include those polypeptides which are produced exclusively or at higher levels in hyperproliferative cells as compared to normal cells. Target antigens include polypeptides encoded by oncogenes such as myb, myc, fyn, and the translocation gene bcr/abl, ras, src, P53, neu, trk and EGRF. In addition to oncogene products as target antigens, target polypeptides for anti-cancer treatments and protective regimens include variable regions of antibodies made by B cell lymphomas and variable regions of T cell receptors of T cell lymphomas which, in some embodiments, are also used as target antigens for autoimmune disease. Other tumor-associated polypeptides can be used as target polypeptides such as polypeptides which are found at higher levels in tumor cells including the polypeptide recognized by monoclonal antibody 17-1A and folate binding polypeptides.

Other suitable therapeutic polypeptides and proteins include those which may be useful for treating individuals suffering from autoimmune diseases and disorders by conferring a broad based protective immune response against targets that are associated with autoimmunity including cell receptors and cells which produce "self"-directed antibodies. T cell mediated autoimmune diseases include Rheumatoid arthritis (RA), multiple sclerosis (MS), Sjogren's syndrome, sarcoidosis, insulin dependent diabetes mellitus (IDDM), autoimmune thyroiditis, reactive arthritis, ankylosing spondylitis, scleroderma, polymyositis, dermatomyositis, psoriasis, vasculitis, Wegener's granulomatosis, Crohn's disease and ulcerative colitis. Each of these diseases is characterized by T cell receptors (TCRs) that bind to endogenous antigens and initiate the inflammatory cascade associated with autoimmune diseases.

Vectors reported herein may be formulated in a manner which permits the expression of a protein carried by the vectors to induce an immune response to a selected antigen. For example, in order to

promote an immune response, the antigen may be expressed from a promoter disclosed herein, the vector can be adjuvanted as described herein, and/or the vector can be put into degenerating tissue.

Examples of suitable immunogenic antigens include those selected from a variety of viral families. Example of desirable viral families against which an immune response would be desirable include, the picornavirus family, which includes the genera rhinoviruses, which are responsible for about 50% of cases of the common cold; the genera enteroviruses, which include polioviruses, coxsackieviruses, echoviruses, and human enteroviruses such as hepatitis A virus; and the genera apthoviruses, which are responsible for foot and mouth diseases, primarily in non-human animals. Within the picornavirus family of viruses, target antigens include the VP1, VP2, VP3, VP4, and VPG. Other viral families include the astroviruses and the calcivirus family. The calcivirus family encompasses the Norwalk group of viruses, which are an important causative agent of epidemic gastroenteritis. Still another viral family desirable for use in targeting antigens for inducing immune responses in humans and non-human animals is the togavirus family, which includes the genera alphavirus, which include Sindbis viruses, RossRiver virus, and Venezuelan, Eastern & Western Equine encephalitis, and rubivirus, including Rubella virus. The flaviviridae family includes dengue, yellow fever, Japanese encephalitis, St. Louis encephalitis and tick borne encephalitis viruses. Other target antigens may be generated from the Hepatitis C or the coronavirus family, which includes a number of non-human viruses such as infectious bronchitis virus (poultry), porcine transmissible gastroenteric virus (pig), porcine hemagglutinatin encephalomyelitis virus (pig), feline infectious peritonitis virus (cats), feline enteric coronavirus (cat), canine coronavirus (dog), and human respiratory coronaviruses, which may cause the common cold and/or non-A, B or C hepatitis, and which include the putative cause of sudden acute respiratory syndrome (SARS). Within the coronavirus family, target antigens include the E1 (also called M or matrix protein), E2 (also called S or Spike protein), E3 (also called HE or hemagglutin-elterose) glycoprotein (not present in all coronaviruses), or N (nucleocapsid). Still other antigens may be targeted against the arterivirus family and the rhabdovirus family. The rhabdovirus family includes the genera vesiculovirus (*e.g.*, Vesicular Stomatitis Virus), and the general lyssavirus (*e.g.*, rabies). Within the rhabdovirus family, suitable antigens may be derived from the G protein or the N protein. The family filoviridae, which includes hemorrhagic fever viruses such as Marburg and Ebola virus may be a suitable source of antigens. The paramyxovirus family includes parainfluenza Virus Type 1, parainfluenza Virus Type 3, bovine parainfluenza Virus Type 3, rubulavirus (mumps virus, parainfluenza Virus Type 2, parainfluenza virus Type 4, Newcastle disease virus (chickens), rinderpest, morbillivirus, which includes measles and canine distemper, and pneumovirus, which includes respiratory syncytial virus. The influenza virus is classified within the family orthomyxovirus and is a suitable source of antigen (*e.g.*, the HA protein, the N1 protein). The bunyavirus family includes the genera bunyavirus (California encephalitis, La Crosse), phlebovirus (Rift Valley Fever), hantavirus (puremala is a hemahagin fever virus), nairovirus (Nairobi sheep disease) and various unassigned bungaviruses. The arenavirus family provides a source of antigens against LCM and Lassa fever virus. Another source of antigens is the bornavirus family. The reovirus family includes the genera reovirus, rotavirus (which causes acute gastroenteritis in children), orbiviruses, and cultivirus (Colorado Tick fever, Lebombo (humans), equine encephalosis, blue tongue). The retrovirus family includes the sub-

family oncorivirinal which encompasses such human and veterinary diseases as feline leukemia virus, HTLVI and HTLVII, lentivirinal (which includes HIV, simian immunodeficiency virus, feline immunodeficiency virus, equine infectious anemia virus, and spumavirinal). The papovavirus family includes the sub-family polyomaviruses (BKU and JCU viruses) and the sub-family papillomavirus (associated with cancers or malignant progression of papilloma). The adenovirus family includes viruses (EX, AD7, ARD, O.B.) which cause respiratory disease and/or enteritis. The parvovirus family feline parvovirus (feline enteritis), feline panleucopeniavirus, canine parvovirus, and porcine parvovirus. The herpesvirus family includes the sub-family alphaherpesvirinae, which encompasses the genera simplexvirus (HSVI, HSVII), varicellovirus (pseudorabies, varicella zoster) and the sub-family betaherpesvirinae, which includes the genera cytomegalovirus (HCMV, muromegalovirus) and the sub-family gamma herpesvirinae, which includes the genera lymphocryptovirus, EBV (Burkitts lymphoma), human herpesviruses 6A, 6B and 7, Kaposi's sarcoma-associated herpesvirus and cercopithecine herpesvirus (B virus), infectious rhinotracheitis, Marek's disease virus, and rhadinovirus. The poxvirus family includes the sub-family chordopoxvirinae, which encompasses the genera orthopoxvirus (Variola major (Smallpox) and Vaccinia (Cowpox)), parapoxvirus, avipoxvirus, capripoxvirus, leporipoxvirus, suipoxvirus, and the sub-family entomopoxvirinae. The hepadnavirus family includes the Hepatitis B virus. One unclassified virus which may be suitable source of antigens is the Hepatitis delta virus, Hepatitis E virus, and prions. Another virus which is a source of antigens is Nipan Virus. Still other viral sources may include avian infectious bursal disease virus and porcine respiratory and reproductive syndrome virus. The alphavirus family includes equine arteritis virus and various Encephalitis viruses.

The present disclosure may also encompass protein based immunogens which are useful to immunize a human or non-human animal against other pathogens including bacteria, fungi, parasitic microorganisms or multicellular parasites which infect human and non-human vertebrates, or from a cancer cell or tumor cell. Examples of bacterial pathogens include pathogenic gram-positive cocci include pneumococci; staphylococci (and the toxins produced thereby, *e.g.*, enterotoxin B); and streptococci. Pathogenic gram-negative cocci include meningococcus; gonococcus. Pathogenic enteric gram-negative bacilli include enterobacteriaceae; pseudomonas, acinetobacteria and eikenella; melioidosis; salmonella; shigella; haemophilus; moraxella; H. ducreyi (which causes chancroid); brucella species (brucellosis); Francisella tularensis (which causes tularemia); Yersinia pestis (plague) and other Yersinia (pasteurella); streptobacillus moniliformis and spirillum; Gram-positive bacilli include listeria monocytogenes; erysipelothrix rhusiopathiae; Corynebacterium diphtheria (diphtheria); cholera; B. anthracia (anthrax); donovanosis (granuloma inguinale); and bartonellosis. Diseases caused by pathogenic anaerobic bacteria include tetanus; botulism (Clostridum botulinum and its toxin); Clostridium perfringens and its epsilon toxin; other clostridia; tuberculosis; leprosy; and other mycobacteria. Pathogenic spirochetal diseases include syphilis; treponematoses: yaws, pinta and endemic syphilis; and leptospirosis. Other infections caused by higher pathogen bacteria and pathogenic fungi include glanders (Burkholderia mallei); actinomycosis; nocardiosis; cryptococcosis, blastomycosis, histoplasmosis and coccidioidomycosis; candidiasis, aspergillosis, and mucormycosis; sporotrichosis; paracoccidiodomycosis, petriellidiosis, torulopsosis, mycetoma and chromomycosis; and dermatophytosis. Rickettsial infections include Typhus

fever, Rocky Mountain spotted fever, Q fever (Coxiella burnetti), and Rickettsialpox. Examples of mycoplasma and chlamydial infections include: mycoplasma pneumoniae; lymphogranuloma venereum; psittacosis; and perinatal chlamydial infections. Pathogenic eukaryotes encompass pathogenic protozoans and helminths and infections produced thereby include: amebiasis; malaria; leishmaniasis; trypanosomiasis; toxoplasmosis; Pneumocystis carinii; Trichans; Toxoplasma gondii; babesiosis; giardiasis; trichinosis; filariasis; schistosomiasis; nematodes; trematodes or flukes; and cestode (tapeworm) infections.

Many of these organisms and/or the toxins produced thereby have been identified by the Centers for Disease Control [(CDC), Department of Health and Human Services, USA], as agents which have potential for use in biological attacks. For example, some of these biological agents, include, Bacillus anthracia (anthrax), Clostridium botulinum and its toxin (botulism), Yersinia pestis (plague), variola major (smallpox), Francisella tularensis (tularemia), and viral hemorrhagic fevers [filoviruses (*e.g.*, Ebola, Marburg], and arenaviruses [*e.g.*, Lassa, Machupo]), all of which are currently classified as Category A agents; Coxiella burnetti (Q fever); Brucella species (brucellosis), Burkholderia mallei (glanders), Burkholderia pseudomallei (meloidosis), Ricinus communis and its toxin (ricin toxin), Clostridium perfringens and its toxin (epsilon toxin), Staphylococcus species and their toxins (enterotoxin B), Chlamydia psittaci (psittacosis), water safety threats (*e.g.*, Vibrio cholerae, Crytosporidium parvum), Typhus fever (Richettsia powazekii), and viral encephalitis (alphaviruses, *e.g.*, Venezuelan equine encephalitis; eastern equine encephalitis; western equine encephalitis); all of which are currently classified as Category B agents; and Nipan virus and hantaviruses, which are currently classified as Category C agents. In addition, other organisms, which are so classified or differently classified, may be identified and/or used for such a purpose in the future. It will be readily understood that the viral vectors and other constructs described herein are useful to deliver antigens from these organisms, viruses, their toxins or other by-products, which will prevent and/or treat infection or other adverse reactions with these biological agents.

In certain embodiments, a protein for expression in a vector of the disclosure are a segment of a variable region of T cells eliciting an immune response, *i.e.*, to eliminate cytotoxic T cells. In rheumatoid arthritis (RA), several specific variable regions of TCRs which are involved in the disease have been characterized. These TCRs include V-3, V-14, V-17 and V-17. Thus, delivery of a nucleic acid sequence that encodes at least one of these polypeptides will elicit an immune response that will target T cells involved in RA. In multiple sclerosis (MS), several specific variable regions of TCRs which are involved in the disease have been characterized. These TCRs include V-7 and V-10. Thus, delivery of a nucleic acid sequence that encodes at least one of these polypeptides will elicit an immune response that will target T cells involved in MS. In scleroderma, several specific variable regions of TCRs which are involved in the disease have been characterized. These TCRs include V-6, V-8, V-14 and V-16, V-3C, V-7, V-14, V-15, V-16, V-28 and V-12. Thus, delivery of a nucleic acid molecule that encodes at least one of these polypeptides will elicit an immune response that will target T cells involved in scleroderma.

Recombinant viral vectors of the disclosure provides an efficient gene transfer vehicle which can deliver a selected proteins to a selected host cell *in vivo* or ex vivo even where the organism has

neutralizing antibodies to the protein. In one embodiment, the vectors disclosed herein and the cells are mixed ex vivo; the infected cells are cultured using conventional methodologies; and the transduced cells are re-infused into the patient.

Additional embodiments

In certain embodiments, the disclosure relates to recombinant viral vectors comprising a liver specific promotor nucleic acid sequence in operable combination with a heterologous nucleic acid sequence encoding a protein. Typically, the nucleic acid sequence encoding the protein comprises a higher percentage of liver cell specific amino acid codons compared to overall human codon usage. In certain embodiments, the disclosure relates to methods of treating a subject diagnosed with a genetic trait that results in expression of a mutated or truncated non-functional protein by administering an effective amount of a vector disclosed herein configured to express a functional protein from the liver.

In certain embodiments, the vector comprises a viral nucleic acid sequence of greater than 10, 20, 30, 40, 50, 100, or 200 nucleotides. In certain embodiments, the viral nucleic acid sequence is a segment of human adeno-associated virus (hAAV) of serotypes 1, 2, 3B, 4, 5, 6, 7, 8, 9 or combinations or variants thereof, typically comprising an AAV inverted terminal repeat.

In certain embodiments, the disclosure relates to a viral particle, *e.g.*, capsid comprising a vector disclosed herein, *e.g.*, the vector packaged in a capsid. The capsid may be a recombinant or chimeric particle or capsid, *e.g.*, capsid having amino acid sequences that are a combination of AAV pseudotypes for VP 1, 2, or 3. An AAV capsid VP may be derived from a human gene or animal AAV gene, or combinations with genetically engineered alterations, *i.e.*, AAV isolated from infected human cells or a non-human primate. Animal AAV include those derived from avian, bovine, porcine, mice, etc. In certain embodiments, the capsid may have amino acid sequences that are bioengineered or synthetic capsids identified through methods such as directed evolution or rational design.

In certain embodiments, vectors disclosed herein are a single or double stranded nucleic acid or self- complementary nucleic acid of less than 5.1, 5.0, 4.9, 4.8, 4.7, 4.6, or 4.5 kb of nucleotides in total. In certain embodiments, the vector is replication-incompetent inside a human host, *e.g.*, vector does not encode a viral polymerase.

In certain embodiments, the liver specific promotor sequence comprises a sequence having at least 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to
GTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTA (SEQ ID NO: 21)
ATAATCTCAGGACAAACA (SEQ ID NO: 43) and/or
TATAAAAGGCCAGCAGCAGCCTGACCACATCTCATCCTC (SEQ ID NO: 20).

In certain embodiments, SEQ ID NO 20 is 3' or after SEQ ID NO: 21 between a nucleotide linker, *e.g.*, connected with a linker as illustrated: 5'- SEQ ID NO: 21 followed by a linker followed by SEQ ID NO: 20 -3'. The linker may be between 0 to 200 nucleotides, 10 to 100 nucleotides, 20 to 70 nucleotides, 30 to 60 nucleotides, 30 to 40 nucleotides, 32 to 36 nucleotides. In certain embodiments, the liver specific promotor sequence comprises a sequence having at least 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to SEQ ID NO: 4, 5, 6, or 7.

As used herein, the liver specific promotor refers to the sequences in the 5' direction of the transcriptional start site of the protein to be produced. Promotor sequences disclosed herein may contain combinations of other known promotors, enhancers, other sequences, and fragments thereof. For example, SEQ ID NO: 21 is the sequence of the shortened ABP enhancer. By itself it does not function as a promoter, it serves to enhance the expression conferred by the core promoter, SEQ ID NO: 20, SynO, which by itself does not confer efficient gene expression. Additional enhancer sequences which may replace or augment the short ABP enhancer are contemplated.

In certain embodiments, the liver specific promotor sequence comprises a sequence having at least 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to CGGAGGAGCAAACAGGG (SEQ ID NO: 97) and/or TATAAAAGGCCAGCAGCAGCCTGACCACATCTCATCCTC (SEQ ID NO: 20).

In certain embodiments, SEQ ID NO 20 is 3' or after SEQ ID NO: 97 between a nucleotide linker, *e.g.*, connected with a linker as illustrated: 5'- SEQ ID NO: 97 followed by a linker followed by SEQ ID NO: 20 -3'. The linker may be between 0 to 200 nucleotides, 10 to 100 nucleotides, 20 to 70 nucleotides, 30 to 60 nucleotides, 30 to 40 nucleotides, 32 to 36 nucleotides.

In certain embodiments, the liver specific promotor sequence comprises a sequence having at least 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to CGGAGGAGCAAACAGGGGCTAAGTCCAC (SEQ ID NO: 98) and/or TATAAAAGGCCAGCAGCAGCCTGACCACATCTCATCCTC (SEQ ID NO: 20).

In certain embodiments, SEQ ID NO 20 is 3' or after SEQ ID NO: 98 between a nucleotide linker, *e.g.*, connected with a linker as illustrated: 5'- SEQ ID NO: 98 followed by a linker followed by SEQ ID NO: 20 -3'. The linker may be between 0 to 200 nucleotides, 10 to 100 nucleotides, 20 to 70 nucleotides, 30 to 60 nucleotides, 30 to 40 nucleotides, 32 to 36 nucleotides.

In certain embodiments, the liver specific promotor sequence comprises a sequence having at least 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to GGCTGCTGGTGAATATTAACCAAGGTC (SEQ ID NO: 99) and/or TATAAAAGGCCAGCAGCAGCCTGACCACATCTCATCCTC (SEQ ID NO: 20).

In certain embodiments, SEQ ID NO 20 is 3' or after SEQ ID NO: 99 between a nucleotide linker, *e.g.*, connected with a linker as illustrated: 5'- SEQ ID NO: 99 followed by a linker followed by SEQ ID NO: 20 -3'. The linker may be between 0 to 200 nucleotides, 10 to 100 nucleotides, 20 to 70 nucleotides, 30 to 60 nucleotides, 30 to 40 nucleotides, 32 to 36 nucleotides.

In certain embodiments, the liver specific promotor sequence comprises a sequence having at least 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to GGGGAGGCTGCTGGTGAATATTAACCAAGGTCACCCCAGTTATCGGAGGAGCAAACAGGGG CTAAGTCCAC (SEQ ID NO: 100) and/or TATAAAAGGCCAGCAGCAGCCTGACCACATCTCATCCTC (SEQ ID NO: 20).

In certain embodiments, SEQ ID NO 20 is 3' or after SEQ ID NO: 100 between a nucleotide linker, *e.g.*, connected with a linker as illustrated: 5'- SEQ ID NO: 100 followed by a linker followed by

SEQ ID NO: 20 -3'. The linker may be between 0 to 200 nucleotides, 10 to 100 nucleotides, 20 to 70 nucleotides, 30 to 60 nucleotides, 30 to 40 nucleotides, 32 to 36 nucleotides.

In certain embodiments, the liver specific promotor sequence comprises a sequence having at least 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to GGGGAGGCTGCTGGTGAATATTAACCAAGGTCACCCCAGTTATCGGAGGAGCAAACAGGGACTAAGTCCAC (SEQ ID NO: 101) and/or TATAAAAGGCCAGCAGCAGCCTGACCACATCTCATCCTC (SEQ ID NO: 20).

In certain embodiments, SEQ ID NO 20 is 3' or after SEQ ID NO: 101 between a nucleotide linker, *e.g.*, connected with a linker as illustrated: 5'- SEQ ID NO: 101 followed by a linker followed by SEQ ID NO: 20 -3'. The linker may be between 0 to 200 nucleotides, 10 to 100 nucleotides, 20 to 70 nucleotides, 30 to 60 nucleotides, 30 to 40 nucleotides, 32 to 36 nucleotides.

In certain embodiments, the liver specific promotor sequence comprises a sequence having at least 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to SEQ ID NO: 4, 5, 6, 7, 102, 103, 104, 105, 106, 107, or 108.

In certain embodiments, liver specific promotor nucleic acid sequence is of less than 205 or 250 nucleotides. The terms "less than 205 or 250 nucleotides" refers to the length of a single strand or the length of double stranded base pairs. The functional promoter is double stranded after intracellular conversion following viral infection. It would not function if it were single stranded.

In certain embodiments, the disclosure contemplates the first nucleotide of the promotor sequence is the 5' "G" in the HFN1a TF binding site, GTTAAT (SEQ ID NO: 25), *e.g.*, promotor is 5' - SEQ ID NO: 21 which is followed by a linker and further followed by a transcriptional start site (TSS), *e.g.* TCATCCTC (SEQ ID NO: 109), wherein the last nucleotide in the transcriptional start site is the end of the promotor sequence. In certain embodiments the linker comprises a TATAA (SEQ ID NO: 26) box and a GC rich spacer. In certain embodiments, the disclosure contemplates the first nucleotide of the promotor sequence is a 5' "G" in GTTAA (SEQ ID NO: 27), GTTA (SEQ ID NO: 28), GTT (SEQ ID NO: 29). In certain embodiments, the first nucleotide of the promotor sequence is a 5' "T" in TTAAT (SEQ ID NO: 30), TTAA (SEQ ID NO: 31), TTA (SEQ ID NO: 32). In certain embodiments, the first nucleotide of the promotor sequence is a 5' "A" in AAT (SEQ ID NO: 33).

In certain embodiments, the disclosure contemplates a promotor comprising 5'-SEQ ID NO: 21 or 97 or 98 or 99 or 100 or 101 optionally followed by a linker, followed by a TATAA box, followed by a GC rich spacer followed by and a transcriptional start site. In certain embodiments, the GC rich spacer is a sequence wherein greater than 60, 70, 80, or 90% of the nucleotides are G or C, *e.g.*, over a window of 5 to 35 nucleotide, 10 to 30 nucleotides, or 15 to 40 nucleotide, or 5 to 50 or 60 nucleotides. In certain embodiments, the CG rich spacer is GGCCAGCAGCAGCCTGACCACATC (SEQ ID NO: 110). In certain embodiments, the liver specific promotor sequence comprises a sequence having at least 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to SEQ ID NO: 110.

In certain embodiments, the liver specific promotor sequence comprises a sequence having at least 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to one of:

SEQ ID NO: 34: GTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACA;

SEQ ID NO: 35: TTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACA;
SEQ ID NO: 36: TAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACA;
SEQ ID NO: 37: AATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACA;
SEQ ID NO: 38: ATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACA;
SEQ ID NO: 39: TTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACA;
SEQ ID NO: 40: TTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACA;
SEQ ID NO: 41: TTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACA;
SEQ ID NO: 42: TTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACA;
SEQ ID NO: 44: TGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACA;
SEQ ID NO: 45: GTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACA;
SEQ ID NO: 46: TGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACA;
SEQ ID NO: 47: GCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACA;
SEQ ID NO: 48: CCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACA;
SEQ ID NO: 49: CCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACA;
SEQ ID NO: 50: CTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACA;
SEQ ID NO: 51: GTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAAC;
SEQ ID NO: 52: GTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAA;
SEQ ID NO: 53: GTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAA;
SEQ ID NO: 54: GTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAA;
SEQ ID NO: 55: GTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACA;
SEQ ID NO: 56: GTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGAC;
SEQ ID NO: 57: GTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGA;
SEQ ID NO: 58: GTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGG;
SEQ ID NO: 59: GTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAG;
SEQ ID NO: 60: GTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCA;
SEQ ID NO: 61: GTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTC;
SEQ ID NO: 62: GTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCT;
SEQ ID NO: 63: GTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATC;
SEQ ID NO: 64: GTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAAT;
SEQ ID NO: 65: GTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAA;
SEQ ID NO: 66: GTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAAC;
SEQ ID NO: 67: TTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAA;
SEQ ID NO: 68: TAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAA;
SEQ ID NO: 69: AATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACA;
SEQ ID NO: 70: ATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGAC;
SEQ ID NO: 71: TTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGA;
SEQ ID NO: 72: TTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGG;
SEQ ID NO: 73: TTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAG;
SEQ ID NO: 74: TTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCA;
SEQ ID NO: 75: TGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTC;
SEQ ID NO: 76: GTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCT;
SEQ ID NO: 77: TGGCCCTTGCGATGTTTGCTCTGGTTAATAATC;
SEQ ID NO: 78: GCCCTTGCGATGTTTGCTCTGGTTAATAAT;
SEQ ID NO: 79: CCCTTGCGATGTTTGCTCTGGTTAATAA;
SEQ ID NO: 80: CCTTGCGATGTTTGCTCTGGTTAATA;
SEQ ID NO: 81: CTTGCGATGTTTGCTCTGGTTAAT;
SEQ ID NO: 82: TTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAAC;
SEQ ID NO: 83: TAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAA;
SEQ ID NO: 84: AATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAA;
SEQ ID NO: 85: ATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAA;
SEQ ID NO: 86: TTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACA;
SEQ ID NO: 87: TTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGAC;
SEQ ID NO: 88: TTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGA;
SEQ ID NO: 89: TTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGG;
SEQ ID NO: 90: TGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAG;
SEQ ID NO: 91: TGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCA;
SEQ ID NO: 92: GGCCCTTGCGATGTTTGCTCTGGTTAATAATCTC;
SEQ ID NO: 93: GCCCTTGCGATGTTTGCTCTGGTTAATAATCT;
SEQ ID NO: 94: CCCTTGCGATGTTTGCTCTGGTTAATAATC;
SEQ ID NO: 95: CCTTGCGATGTTTGCTCTGGTTAATAAT; or
SEQ ID NO: 96: CTTGCGATGTTTGCTCTGGTTAATAA.

In certain embodiments, the promotor may start with the first 5' nucleotide of SEQ ID NO: 34-96, and end with the last nucleotide of the TSS.

In certain embodiments, the protein is a fVIII or fIX or variant thereof. In certain embodiments, the promoter and codon optimization schemes disclosed herein could be used for any liver-directed AAV gene therapies. Other metabolic diseases caused by deficiencies of liver enzymes and expression of those functional proteins are contemplated.

In certain embodiments, fVIII variant comprises an A1 domain, an A2 domain, a RHQR sequence (SEQ ID NO: 24), an A3 domain, a C1 domain, and a C2 domain. In certain embodiments, the fVIII variant comprises a deleted B domain.

In certain embodiments, the fVIII variant comprises a linker of between two and fifty, or two and twenty five, or two and fifteen amino acids between the A2 domain and the A3 domain.

In certain embodiments, fVIII variant comprises an A1 domain, an A2 domain, an activation peptide (ap) domain, an A3 domain, a C1 domain, and a C2 domain. In certain embodiments, the fVIII variant comprises a deleted B domain.

In certain embodiments, the fVIII variant comprises a linker of between two and fifty, or two and twenty five, or two and fifteen amino acids between the A2 domain and the an activation peptide (ap) domain.

In certain embodiments, the fVIII variant comprises a sequence having at least 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to SEQ ID NO: 3.

In certain embodiments, the fVIII variant comprises a sequence having at least 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to SEQ ID NO: 13, 14, 15, or 16.

In certain embodiments, the disclosure relates to methods wherein codon usage of a gene is adjusted according to the tissue it will be expressed, *e.g.*, liver tissue. In certain embodiments, the nucleic acid sequence encoding a protein comprises codons that are differentially utilized or represented in genes highly expressed within the liver or other specific tissue compared to the codon usage of the entire coding region of the human genome and avoids codons that are under-represented in the liver or other specific tissue.

In certain embodiments, the nucleic acid sequence encoding the protein comprises codons for greater than 50, 60, 70, 80, 90, or 95% or 100% of the amino acids that are preferred as provided in FIG 2.

In certain embodiments, the nucleic acid sequence encoding a protein comprises a higher percentage of liver cell specific amino acid codons compared to overall human codon usage is a sequence having at least 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to SEQ ID NO: 1.

In certain embodiments, fIX variant comprises a sequence having at least 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to SEQ ID NO: 17, 18, or 19.

In certain embodiments, the nucleic acid sequence encoding a protein comprising a higher percentage of liver cell specific amino acid codons compared to overall human codon usage is a sequence having at least 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, or 100% sequence identity to SEQ ID NO: 8, 9, or 10.

In certain embodiments, the nucleic acid sequence encoding the protein comprises at least one of a) to g) wherein,

a) a ATC codon is in greater than 50 % or 52 % for Ile;

b) a ACC codon is in greater than 38 % or 40 % for Thr;

c) a TTC codon is in greater than 57% or 59 % for Phe;

d) a GAG codon is in greater than 60% or 62% for Glu;

e) a CTG codon is in greater than 43% or 45% for Leu;

f) a AAG codon is in greater than 60 % or 62 % for Lys; and/or

g) a GAC codon is in greater than 56% or 58 % for Asp.

In certain embodiments, the nucleic acid sequence encoding the protein comprises at least two or more, or three or more, or four or more, five or more, six or more, or all of a), b), c), d), e), f) and g).

In certain embodiments, the nucleic acid sequence encoding the protein comprises less than 100, 50, 30, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2 or no 5'-CG-3' dinucleotides.

In certain embodiments, the disclosure relates to pharmaceutical compositions comprising a vector or a capsid as disclosed herein and a pharmaceutically acceptable excipient.

In certain embodiments, the disclosure relates to methods of inducing blood clotting comprising administering an effective amount of a virus particle, capsid, or vector as disclosed herein to a subject in need thereof.

In certain embodiments, the subject is diagnosed with hemophilia A or B or acquired hemophilia or unlikely to respond to exogenous fVIII infusions.

In certain embodiments, the vector, virus particle, or capsid is administered in combination with an immunosuppressive agent, *e.g.*, ciclosporin, tacrolimus, sirolimus, cyclophosphamide, methotrexate, azathioprine, mercaptopurine, fluorouracil, mycophenolic acid, dactinomycin, fingolimod, T-cell receptor antibody or binding protein, muromonab-CD3, IL-2 receptor antibody or binding protein, basiliximab, daclizumab, recombinant IFN-beta, TNF-alpha antibody or binding protein, infliximab, etanercept, adalimumab, or combinations thereof.

In certain embodiments, the disclosure relates to expression systems comprising nucleic acids or vectors comprising nucleic acids disclosed herein.

In certain embodiments, the disclosure relates to expression systems comprising nucleic acids or vectors comprising nucleic acids disclosed herein.

Additional embodiments are illustrated by the following clauses:

Clause 1. A vector comprising a liver specific promotor nucleic acid sequence of less than 250 nucleotides in operable combination with a heterologous nucleic acid sequence encoding a protein.

Clause 2. The vector of clause 1, wherein the liver specific promotor sequence comprises a sequence having greater than 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, or 99 % sequence identity to GGCCAGCAGCAGCCTGACCACATC (SEQ ID NO: 110).

Clause 3. The vector of clause 1, wherein the liver specific promotor sequence comprises a sequence having greater than 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, or 99 % sequence identity to SEQ ID NO: 4, 5, 6, 7, 102, 103, 104, 105, 106, 107, or 108.

Clause 4. The vector of clause 1, wherein the protein is a fVIII or fIX or variant thereof.

Clause 5. The vector of clause 4, wherein fVIII variant comprises an A1 domain, an A2 domain, an ap domain, an A3 domain, a C1 domain, and a C2 domain

Clause 6. The vector of clause 4, wherein the fVIII variant comprising a deleted B domain.

Clause 7. The vector of clause 4, wherein the fVIII variant comprises a linker of between two and fifty, or two and twenty five, or two and fifteen amino acids between the A2 domain and the an activation peptide(ap) domain.

Clause 8. The vector of clause 4, wherein the fVIII variant comprises a sequence having greater than 50, 60, 70, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 % sequence identity to SEQ ID NO: 3.

Clause 9. The vector of clause 4, wherein the fVIII variant comprises a sequence having greater than 50, 60, 70, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 % sequence identity to SEQ ID NO: 13, 14, 15, or 16.

Clause 10. The method of clause 1 wherein the heterologous nucleic acids sequence comprises a higher percentage of liver cell specific amino acid codons compared to overall human codon usage.

Clause 11. The vector of clause 1, wherein the nucleic acid sequence encoding a protein comprising a higher percentage of liver cell specific amino acid codons compared to overall human codon usage is a sequence having greater than 70, 80, 85, 90, 95, 96, 97, 98, or 99 % sequence identity to SEQ ID NO: 2 or 11.

Clause 12. The vector of clause 3, wherein fIX variant comprises a sequence having greater than 50, 60, 70, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 % sequence identity to SEQ ID NO: 17, 18, or 19.

Clause 13. The vector of clause 1, wherein the nucleic acid sequence encoding a protein comprising a higher percentage of liver cell specific amino acid codons compared to overall human codon usage is a sequence having greater than 70, 80, 85, 90, 95, 96, 97, 98, or 99 % sequence identity to SEQ ID NO: 8, 9, or 10.

Clause 14. A pharmaceutical composition comprising a vector of clause 1 and a pharmaceutically acceptable excipient.

Clause 15. A method of inducing blood clotting comprising administering an effective amount of a vector of clause 1 to a subject in need thereof.

Clause 16. The method of clause 15, wherein the subject is diagnosed with hemophilia A or B or acquired hemophilia.

Clause 17. The method of clause 15, wherein the vector is administered in combination with an immunosuppressive agent.

Clause 18. The method of clause 17, wherein the immunosuppressive agent is ciclosporin, tacrolimus, sirolimus, cyclophosphamide, methotrexate, azathioprine, mercaptopurine, fluorouracil, mycophenolic acid, dactinomycin, fingolimod, T-cell receptor antibody or binding protein, muromonab-

CD3, IL-2 receptor antibody or binding protein, basiliximab, daclizumab, recombinant IFN-beta, TNF-alpha antibody or binding protein, infliximab, etanercept, or adalimumab.

The following examples are provided to illustrate certain particular features and/or embodiments. These examples should not be construed to limit the disclosure to the particular features or embodiments described.

EXAMPLES

Example 1

Optimization of Clotting factors and their encoding DNA

This example illustrates the optimization of the coding sequences for fVIII and fIX proteins to improve their utility for *in vivo* expression and gene therapy.

The cDNA nucleotide sequence coding for fVIII and fIX was initially optimized by implementing a codon usage bias specific for the human liver cell as compared to naturally occurring nucleotide sequence coding for the corresponding non-codon optimized sequence for a human.

The adaptiveness of a nucleotide sequence encoding fVIII to the codon usage of human liver cells may be expressed as liver codon adaptation index (LCAI). A codon adaptation index is defined as a measurement of the relative adaptiveness of the codon usage of a gene towards the codon usage of genes highly expressed in the human liver. The relative adaptiveness of each codon is the ratio of the usage of each codon, to that of the most abundant codon for the same amino acid. The CAI is defined as the geometric mean of these relative adaptiveness values. Non-synonymous codons and termination codons (dependent on genetic code) are excluded. CAI values range from 0 to 1, with higher values indicating a higher proportion of the most abundant codons. Using the sequences of 43 highly expressed genes in the human liver, a custom codon-usage bias table specific for the human liver was constructed (see FIG. 2A) that differs substantially from the most prevalent codons used in total human coding sequences (FIG. 2B). Optimized coding sequence of the ET3 and HSQ variants of fVIII was developed with the most statistically prevalent codons identified by the liver-usage bias analysis.

ET3 is a B domain deleted (BDD) fVIII hybrid that contains human and porcine domains, *i.e.*, sequence (A1 and A3 porcine, see Figure 1A and 1B) with a linker in the deleted B domain. ET3 utilizes a 24 amino acid porcine sequence-derived OL linker sequence, *i.e.*, porcine-derived sequence SFAQNSRPPSASAPKPPVLRRHQR (SEQ ID NO: 23). The ET3 amino acid sequence is SEQ ID NO: 123:

MQLELSTCVFLCLLPLGFSAIRRYYLGAVELSWDYRQSELLRELHVDTRFPATAPGALPLGPSVLYKKTVFVEFTDQL
FSVARPRPPWMGLLGPTIQAEVYDTVVVTLKNMASHPVSLHAVGVSFWKSSEGAEYEDHTSQREKEDDKVLPGKSQTY
VWQVLKENGPTASDPPCLTYSYLSHVDLVKDLNSGLIGALLVCREGSLTRERTQNLHEFVLLFAVFDEGKSWHSARND
SWTRAMDPAPARAQPAMHTVNGYVNRSLPGLIGCHKKSVYWHVIGMGTSPEVHSIFLEGHTFLVRHHRQASLEISPLT
FLTAQTFLMDLGQFLLFCHISSHHHGGMEAHVRVESCAEEPQLRRKADEEEDYDDNLYDSDMDVVRLDGDDVSPFIQI
RSVAKKHPKTWVHYIAAEEEDWDYAPLVLAPDDRSYKSQYLNNGPQRIGRKYKKVRFMAYTDETFKTREAIQHESGIL
GPLLYGEVGDTLLIIFKNQASRPYNIYPHGITDVRPLYSRRLPKGVKHLKDFPILPGEIFKYKWTVTVEDGPTKSDPR
CLTRYYSSFVNMERDLASGLIGPLLICYKESVDQRGNQIMSDKRNVILFSVFDENRSWYLTENIQRFLPNPAGVQLED
PEFQASNIMHSINGYVFDSLQLSVCLHEVAYWYILSIGAQTDFLSVFFSGYTFKHKMVYEDTLTLFPFSGETVFMSME
NPGLWILGCHNSDFRNRGMTALLKVSSCDKNTGDYYEDSYEDISAYLLSKNNAIEPRSFAQNSRPPSASAPKPPVLRR
HQRDISLPTFQPEEDKMDYDDIFSTETKGEDFDIYGEDENQDPRSFQKRTRHYFIAAVEQLWDYGMSESPRALRNRAQ
NGEVPRFKKVVFREFADGSFTQPSYRGELNKHLGLLGPYIRAEVEDNIMVTFKNQASRPYSFYSSLISYPDDQEQGAE

PRHNFVQPNETRTYFWKVQHHMAPTEDEFDCKAWAYFSDVDLEKDVHSGLIGPLLICRANTLNAAHGRQVTVQEFALF
FTIFDETKSWYFTENVERNCRAPCHLQMEDPTLKENYRFHAINGYVMDTLPGLVMAQNQRIRWYLLSMGSNENIHSIH
FSGHVFSVRKKEEYKMAVYNLYPGVFETVEMLPSKVGIWRIECLIGEHLQAGMSTTFLVYSKKCQTPLGMASGHIRDF
QITASGQYGQWAPKLARLHYSGSINAWSTKEPFSWIKVDLLAPMIIHGIKTQGARQKFSSLYISQFIIMYSLDGKKWQ
TYRGNSTGTLMVFFGNVDSSGIKHNIFNPPIIARYIRLHPTHYSIRSTLRMELMGCDLNSCSMPLGMESKAISDAQIT
ASSYFTNMFATWSPSKARLHLQGRSNAWRPQVNNPKEWLQVDFQKTMKVTGVTTQGVKSLLTSMYVKEFLISSSQDGH
QWTLFFQNGKVKVFQGNQDSFTPVVNSLDPPLLTRYLRIHPQSWVHQIALRMEVLGCEAQDLY

HSQ is a human fVIII variant wherein the BDD human fVIII protein is substituted with a 14 amino acid human-derived SQ linker SFSQNPPVLKRHQR (SEQ ID NO: 22). The HSQ amino acid sequence is SEQ ID NO: 3:

MQIELSTCFFLCLLRFCFSATRRYYLGAVELSWDYMQSDLGELPVDARFPPRVPKSFPFNTSVVYKKTLFVEFTVHLF
NIAKPRPPWMGLLGPTIQAEVYDTVVITLKNMASHPVSLHAVGVSYWKASEGAEYDDQTSQREKEDDKVFPGGSHTYV
WQVLKENGPMASDPLCLTYSYLSHVDLVKDLNSGLIGALLVCREGSLAKEKTQTLHKFILLFAVFDEGKSWHSETKNS
LMQDRDAASARAWPKMHTVNGYVNRSLPGLIGCHRKSVYWHVIGMGTTPEVHSIFLEGHTFLVRNHRQASLEISPITF
LTAQTLLMDLGQFLLFCHISSHQHDGMEAYVKVDSCPEEPQLRMKNNEEAEDYDDDLTDSEMDVVRFDDDNSPSFIQI
RSVAKKHPKTWVHYIAAEEEDWDYAPLVLAPDDRSYKSQYLNNGPQRIGRKYKKVRFMAYTDETFKTREAIQHESGIL
GPLLYGEVGDTLLIIFKNQASRPYNIYPHGITDVRPLYSRRLPKGVKHLKDFPILPGEIFKYKWTVTVEDGPTKSDPR
CLTRYYSSFVNMERDLASGLIGPLLICYKESVDQRGNQIMSDKRNVILFSVFDENRSWYLTENIQRFLPNPAGVQLED
PEFQASNIMHSINGYVFDSLQLSVCLHEVAYWYILSIGAQTDFLSVFFSGYTFKHKMVYEDTLTLFPFSGETVFMSME
NPGLWILGCHNSDFRNRGMTALLKVSSCDKNTGDYYEDSYEDISAYLLSKNNAIEPRSFSQNPPVLKRHQREITRTTL
QSDQEEIDYDDTISVEMKKEDFDIYDEDENQSPRSFQKKTRHYFIAAVERLWDYGMSSSPHVLRNRAQSGSVPQFKKV
VFQEFTDGSFTQPLYRGELNEHLGLLGPYIRAEVEDNIMVTFRNQASRPYSFYSSLISYEEDQRQGAEPRKNFVKPNE
TKTYFWKVQHHMAPTKDEFDCKAWAYFSDVDLEKDVHSGLIGPLLVCHTNTLNPAHGRQVTVQEFALFFTIFDETKSW
YFTENMERNCRAPCNIQMEDPTFKENYRFHAINGYIMDTLPGLVMAQDQRIRWYLLSMGSNENIHSIHFSGHVFTVRK
KEEYKMALYNLYPGVFETVEMLPSKAGIWRVECLIGEHLHAGMSTLFLVYSNKCQTPLGMASGHIRDFQITASGQYGQ
WAPKLARLHYSGSINAWSTKEPFSWIKVDLLAPMIIHGIKTQGARQKFSSLYISQFIIMYSLDGKKWQTYRGNSTGTL
MVFFGNVDSSGIKHNIFNPPIIARYIRLHPTHYSIRSTLRMELMGCDLNSCSMPLGMESKAISDAQITASSYFTNMFA
TWSPSKARLHLQGRSNAWRPQVNNPKEWLQVDFQKTMKVTGVTTQGVKSLLTSMYVKEFLISSSQDGHQWTLFFQNGK
VKVFQGNQDSFTPVVNSLDPPLLTRYLRIHPQSWVHQIALRMEVLGCEAQDLY

Both HSQ and ET3 contain the RHQR (SEQ ID NO: 24) recognition sequence for PACE/furin processing sequence for the B-domain.

The liver codon optimized ET3 sequence is (SEQ ID NO: 12)

ATGCAGCTGGAACTGTCTACCTGTGTGTTTCTGTGTCTGCTGCCTCTGGGGTTTTCTGCTATCCGCCGCTACTATCTG
GGAGCCGTGGAGCTGTCCTGGGACTACAGGCAGAGCGAGCTGCTGAGAGAACTGCACGTGGATACCAGATTCCCAGCT
ACCGCTCCAGGAGCTCTGCCTCTGGGCCCATCCGTGCTGTACAAGAAAACCGTCTTCGTGGAGTTTACCGACCAGCTG
TTCAGCGTGGCCAGGCCAAGACCACCTTGGATGGGACTGCTGGGACCAACCATCCAGGCTGAGGTGTACGATACCGTG
GTCGTGACCCTGAAAAACATGGCCTCCCATCCCGTGAGCCTGCACGCTGTCGGGGTGTCCTTCTGGAAGTCCAGCGAG
GGAGCCGAGTACGAAGACCATACCTCCCAGCGCGAGAAAGAAGACGATAAGGTGCTGCCTGGCAAAAGCCAGACCTAT
GTCTGGCAGGTGCTGAAGGAGAACGGACCAACCGCTAGCGACCCACCATGCCTGACCTACTCTTATCTGTCCCACGTC
GATCTGGTGAAGGACCTGAATTCCGGACTGATCGGAGCTCTGCTGGTGTGTAGAGAGGGAAGCCTGACCAGAGAAAGA
ACCCAGAACCTGCATGAGTTCGTCCTGCTGTTCGCCGTGTTTGACGAAGGGAAGAGCTGGCACTCTGCCCGCAATGAC
TCCTGGACCAGAGCTATGGATCCAGCTCCTGCTAGAGCTCAGCCTGCTATGCACACCGTCAACGGCTACGTGAATCGG
TCTCTGCCAGGACTGATCGGCTGCCATAAGAAAAGCGTCTATTGGCACGTGATCGGAATGGGCACCAGCCCCGAGGTG
CATTCTATCTTCCTGGAAGGCCACACCTTTCTGGTCAGGCACCATAGACAGGCCTCTCTGGAGATCTCCCCTCTGACC
TTCCTGACCGCTCAGACCTTTCTGATGGACCTGGGGCAGTTCCTGCTGTTTTGCCATATCTCTTCCCACCATCACGGA
GGAATGGAGGCTCACGTCAGGGTGGAATCCTGTGCTGAGGAACCACAGCTGAGAAGAAAGGCTGATGAGGAAGAGGAC
TACGACGATAACCTGTATGACAGCGATATGGACGTCGTGCGCCTGGACGGCGACGATGTCAGCCCTTTCATCCAGATC
CGGTCTGTGGCCAAGAAACATCCAAAGACCTGGGTCCACTACATCGCCGCTGAAGAGGAAGATTGGGACTATGCCCCC
CTGGTGCTGGCTCCTGACGATAGATCCTACAAAAGCCAGTATCTGAACAATGGGCCCCAGCGCATCGGACGGAAGTAC
AAGAAAGTGAGGTTCATGGCCTATACCGACGAGACCTTTAAGACCAGAGAGGCTATCCAGCACGAATCCGGGATCCTG
GGACCTCTGCTGTACGGCGAAGTGGGGGATACCCTGCTGATCATCTTCAAGAACCAGGCCTCCAGGCCATACAATATC
TATCCCCATGGCATCACCGACGTGAGACCACTGTACAGCAGGAGACTGCCCAAGGGGGTCAAACACCTGAAGGATTTC
CCCATCCTGCCTGGAGAGATCTTTAAGTATAAATGGACCGTCACCGTGGAAGACGGGCCTACCAAGTCCGATCCACGC
TGCCTGACCCGGTACTATAGCTCTTTCGTGAACATGGAGAGAGACCTGGCTAGCGGACTGATCGGACCCCTGCTGATC
TGTTACAAAGAGAGCGTGGACCAGAGGGGCAACCAGATCATGTCTGATAAGAGAAATGTCATCCTGTTCTCCGTGTTT
GACGAGAACCGCAGCTGGTACCTGACCGAGAACATCCAGCGGTTCCTGCCAAATCCAGCTGGAGTGCAGCTGGAGGAC
CCAGAATTTCAGGCTTCCAACATCATGCATAGCATCAATGGCTACGTGTTCGATAGCCTGCAGCTGTCTGTCTGCCTG

CACGAGGTGGCCTACTGGTATATCCTGTCCATCGGCGCTCAGACCGACTTCCTGTCCGTGTTCTTTAGCGGGTACACC
TTTAAGCATAAAATGGTGTATGAGGATACCCTGACCCTGTTCCCCTTTTCTGGCGAGACCGTGTTCATGTCCATGGAA
AACCCTGGCCTGTGGATCCTGGGGTGCCACAACAGCGACTTCAGGAATAGAGGAATGACCGCCCTGCTGAAAGTGTCC
AGCTGTGATAAGAATACCGGCGATTACTATGAGGACTCTTACGAAGATATCTCCGCTTATCTGCTGAGCAAGAACAAT
GCCATCGAGCCCAGGTCTTTCGCTCAGAACTCCAGACCTCCAAGCGCTTCTGCTCCTAAGCCACCTGTGCTGAGAAGA
CATCAGAGGGACATCTCCCTGCCTACCTTCCAGCCAGAGGAAGATAAAATGGACTACGACGATATCTTCAGCACCGAG
ACCAAGGGGGAAGATTTTGACATCTATGGAGAGGACGAAAACCAGGATCCAAGATCCTTCCAGAAGAGAACCAGACAC
TACTTTATCGCCGCTGTGGAGCAGCTGTGGGACTATGGGATGTCCGAAAGCCCACGGGCCCTGAGGAACAGAGCTCAG
AATGGAGAGGTGCCCCGCTTCAAGAAAGTCGTGTTCCGGGAGTTTGCCGACGGCAGCTTTACCCAGCCATCTTACAGG
GGGGAGCTGAACAAGCATCTGGGGCTGCTGGGACCCTATATCAGAGCCGAGGTCGAAGATAACATCATGGTGACCTTC
AAGAATCAGGCTTCTCGCCCCTACTCCTTTTATTCTTCCCTGATCTCCTACCCTGACGATCAGGAGCAGGGCGCCGAA
CCTAGGCACAACTTCGTGCAGCCAAATGAGACCAGAACCTACTTTTGGAAGGTGCAGCATCACATGGCTCCCACCGAG
GATGAATTCGACTGCAAAGCTTGGGCCTATTTTTCCGATGTCGACCTGGAGAAGGACGTGCATAGCGGCCTGATCGGG
CCTCTGCTGATCTGTCGCGCCAACACCCTGAATGCTGCTCACGGAAGACAGGTCACCGTGCAGGAGTTCGCTCTGTTC
TTTACCATCTTTGACGAAACCAAGAGCTGGTACTTCACCGAGAACGTGGAAAGGAATTGCAGAGCCCCCTGTCATCTG
CAGATGGAGGACCCTACCCTGAAGGAAAACTACAGGTTCCACGCCATCAATGGATATGTCATGGATACCCTGCCCGGC
CTGGTCATGGCTCAGAACCAGCGCATCCGGTGGTACCTGCTGTCTATGGGATCCAACGAGAATATCCATAGCATCCAC
TTCTCTGGCCATGTCTTTTCCGTGAGGAAGAAAGAGGAATACAAAATGGCCGTGTACAATCTGTATCCTGGGGTCTTC
GAGACCGTGGAAATGCTGCCAAGCAAAGTGGGAATCTGGAGAATCGAGTGCCTGATCGGCGAACACCTGCAGGCCGGG
ATGAGCACCACCTTCCTGGTGTACTCTAAGAAATGTCAGACCCCACTGGGGATGGCCTCCGGACATATCCGCGACTTC
CAGATCACCGCTAGCGGACAGTACGGACAGTGGGCTCCAAAGCTGGCTAGACTGCACTATTCTGGCTCCATCAACGCC
TGGTCTACCAAAGAGCCATTCTCCTGGATCAAGGTGGACCTGCTGGCCCCCATGATCATCCACGGAATCAAAACCCAG
GGCGCTAGGCAGAAGTTCAGCTCTCTGTACATCTCCCAGTTTATCATCATGTATAGCCTGGACGGGAAGAAATGGCAG
ACCTACAGAGGCAATTCCACCGGGACCCTGATGGTCTTCTTTGGAAACGTGGATTCCAGCGGCATCAAGCACAACATC
TTCAATCCACCCATCATCGCCCGCTACATCCGGCTGCATCCTACCCACTATAGCATCAGGTCTACCCTGAGAATGGAG
CTGATGGGATGCGACCTGAACAGCTGTTCTATGCCACTGGGCATGGAGTCCAAGGCTATCAGCGATGCCCAGATCACC
GCTTCTTCCTACTTCACCAATATGTTTGCTACCTGGTCCCCAAGCAAGGCTAGACTGCACCTGCAGGGAAGATCCAAC
GCTTGGAGACCCCAGGTGAACAATCCTAAGGAGTGGCTGCAGGTCGACTTCCAGAAAACCATGAAGGTCACCGGGGTG
ACCACCCAGGGAGTGAAATCTCTGCTGACCTCCATGTACGTCAAGGAGTTCCTGATCAGCTCTTCCCAGGACGGCCAC
CAGTGGACCCTGTTCTTTCAGAACGGCAAGGTCAAAGTGTTCCAGGGGAATCAGGACTCTTTTACCCCCGTCGTGAAC
TCCCTGGATCCTCCACTGCTGACCAGGTACCTGAGAATCCATCCTCAGAGCTGGGTGCACCAGATCGCTCTGAGAATG
GAGGTCCTGGGATGCGAAGCTCAGGACCTGTATTGA

In addition, CpG DNA motifs in the liver-codon optimized coding sequence for ET3 and HSQ were removed because they may lead to gene methylation and silencing. See Bird, DNA methylation and the frequency of CpG in animal DNA, 1980, Nucleic Acids Res, 8: 1499–1504. Codons were substituted with the most highly used human/ liver alternative (based on the liver-usage bias analysis discussed above) that did not result in the formation of a 5'-CG-3' dinucleotide in the sequence. These modifications removed 174 and 175 CpGs from the liver-codon optimized ET3 and HSQ sequences, respectively. CpG removal also helps the vector evade immune detection, enhancing the safety and efficacy of the vector. See J Clin Invest. 2013, 123(7):2994-3001, entitled "CpG-depleted adeno-associated virus vectors evade immune detection."

The CpG deleted, liver codon optimized ET3 sequence is SEQ ID NO: 11:

ATGCAGCTGGAACTGTCTACCTGTGTGTTTCTGTGTCTGCTGCCTCTGGGGTTTTCTGCTATCAGGAGATACTATCTG
GGAGCTGTGGAGCTGTCCTGGGACTACAGGCAGTCTGAGCTGCTGAGAGAACTGCATGTGGATACCAGATTCCCAGCT
ACAGCTCCAGGAGCTCTGCCTCTGGGCCCATCTGTGCTGTACAAGAAAACAGTCTTTGTGGAGTTTACAGACCAGCTG
TTCTCTGTGGCCAGGCCAAGACCACCTTGGATGGGACTGCTGGGACCAACCATCCAGGCTGAGGTGTATGATACAGTG
GTGGTGACCCTGAAAAACATGGCCTCCCATCCTGTGAGCCTGCATGCTGTGGGGGTGTCCTTCTGGAAGTCCTCTGAG
GGAGCTGAGTATGAAGACCATACCTCCCAGAGGGAGAAAGAAGATGATAAGGTGCTGCCTGGCAAAAGCCAGACCTAT
GTCTGGCAGGTGCTGAAGGAGAATGGACCAACTGCTTCTGACCCACCATGCCTGACCTACTCTTATCTGTCCCATGTG
GATCTGGTGAAGGACCTGAATTCTGGACTGATTGGAGCTCTGCTGGTGTGTAGAGAGGGAAGCCTGACCAGAGAAAGA
ACCCAGAACCTGCATGAGTTTGTCCTGCTGTTTGCTGTGTTTGATGAAGGGAAGAGCTGGCACTCTGCCAGGAATGAC
TCCTGGACCAGAGCTATGGATCCAGCTCCTGCTAGAGCTCAGCCTGCTATGCACACAGTCAATGGCTATGTGAATAGG
TCTCTGCCAGGACTGATTGGCTGCCATAAGAAATCTGTCTATTGGCATGTGATTGGAATGGGCACCAGCCCTGAGGTG
CATTCTATCTTCCTGGAAGGCCACACCTTTCTGGTCAGGCACCATAGACAGGCCTCTCTGGAGATCTCCCCTCTGACC
TTCCTGACAGCTCAGACCTTTCTGATGGACCTGGGGCAGTTCCTGCTGTTTTGCCATATCTCTTCCCACCATCATGGA
GGAATGGAGGCTCATGTCAGGGTGGAATCCTGTGCTGAGGAACCACAGCTGAGAAGAAAGGCTGATGAGGAAGAGGAC

TATGATGATAACCTGTATGACTCTGATATGGATGTGGTGAGGCTGGATGGGGATGATGTCAGCCCTTTCATCCAGATC
AGGTCTGTGGCCAAGAAACATCCAAAGACCTGGGTCCACTACATTGCTGCTGAAGAGGAAGATTGGGACTATGCCCCC
CTGGTGCTGGCTCCTGATGATAGATCCTACAAAAGCCAGTATCTGAACAATGGGCCCCAGAGGATTGGAAGGAAGTAC
AAGAAAGTGAGGTTCATGGCCTATACAGATGAGACCTTTAAGACCAGAGAGGCTATCCAGCATGAATCTGGGATCCTG
GGACCTCTGCTGTATGGAGAAGTGGGGGATACCCTGCTGATCATCTTCAAGAACCAGGCCTCCAGGCCATACAATATC
TATCCCCATGGCATCACAGATGTGAGACCACTGTACAGCAGGAGACTGCCCAAGGGGGTCAAACACCTGAAGGATTTC
CCCATCCTGCCTGGAGAGATCTTTAAGTATAAATGGACAGTCACAGTGGAAGATGGGCCTACCAAGTCTGATCCAAGG
TGCCTGACCAGATACTATAGCTCTTTTGTGAACATGGAGAGAGACCTGGCTTCTGGACTGATTGGACCCCTGCTGATC
TGTTACAAAGAGTCTGTGGACCAGAGGGGCAACCAGATCATGTCTGATAAGAGAAATGTCATCCTGTTCTCTGTGTTT
GATGAGAACAGGAGCTGGTACCTGACAGAGAACATCCAGAGGTTCCTGCCAAATCCAGCTGGAGTGCAGCTGGAGGAC
CCAGAATTTCAGGCTTCCAACATCATGCATAGCATCAATGGCTATGTGTTTGATAGCCTGCAGCTGTCTGTCTGCCTG
CATGAGGTGGCCTACTGGTATATCCTGTCCATTGGAGCTCAGACAGACTTCCTGTCTGTGTTCTTTAGTGGGTACACC
TTTAAGCATAAAATGGTGTATGAGGATACCCTGACCCTGTTCCCCTTTTCTGGGGAGACAGTGTTCATGTCCATGGAA
AACCCTGGCCTGTGGATCCTGGGGTGCCACAACTCTGACTTCAGGAATAGAGGAATGACAGCCCTGCTGAAAGTGTCC
AGCTGTGATAAGAATACAGGGGATTACTATGAGGACTCTTATGAAGATATCTCTGCTTATCTGCTGAGCAAGAACAAT
GCCATTGAGCCCAGGTCTTTTGCTCAGAACTCCAGACCTCCATCTGCTTCTGCTCCTAAGCCACCTGTGCTGAGAAGA
CATCAGAGGGACATCTCCCTGCCTACCTTCCAGCCAGAGGAAGATAAAATGGACTATGATGATATCTTCAGCACAGAG
ACCAAGGGGGAAGATTTTGACATCTATGGAGAGGATGAAAACCAGGATCCAAGATCCTTCCAGAAGAGAACCAGACAC
TACTTTATTGCTGCTGTGGAGCAGCTGTGGGACTATGGGATGTCTGAAAGCCCAAGGGCCCTGAGGAACAGAGCTCAG
AATGGAGAGGTGCCCAGATTCAAGAAAGTGGTGTTCAGAGAGTTTGCTGATGGCAGCTTTACCCAGCCATCTTACAGG
GGGGAGCTGAACAAGCATCTGGGGCTGCTGGGACCCTATATCAGAGCTGAGGTGGAAGATAACATCATGGTGACCTTC
AAGAATCAGGCTTCTAGGCCCTACTCCTTTTATTCTTCCCTGATCTCCTACCCTGATGATCAGGAGCAGGGAGCTGAA
CCTAGGCACAACTTTGTGCAGCCAAATGAGACCAGAACCTACTTTTGGAAGGTGCAGCATCACATGGCTCCCACAGAG
GATGAATTTGACTGCAAAGCTTGGGCCTATTTTTCTGATGTGGACCTGGAGAAGGATGTGCATTCTGGCCTGATTGGG
CCTCTGCTGATCTGTAGGGCCAACACCCTGAATGCTGCTCATGGAAGACAGGTCACAGTGCAGGAGTTTGCTCTGTTC
TTTACCATCTTTGATGAAACCAAGAGCTGGTACTTCACAGAGAATGTGGAAAGGAATTGCAGAGCCCCCTGTCATCTG
CAGATGGAGGACCCTACCCTGAAGGAAAACTACAGGTTCCATGCCATCAATGGATATGTCATGGATACCCTGCCTGGC
CTGGTCATGGCTCAGAACCAGAGGATCAGATGGTACCTGCTGTCTATGGGATCCAATGAGAATATCCATAGCATCCAC
TTCTCTGGCCATGTCTTTTCTGTGAGGAAGAAAGAGGAATACAAAATGGCTGTGTACAATCTGTATCCTGGGGTCTTT
GAGACAGTGGAAATGCTGCCAAGCAAAGTGGGAATCTGGAGAATTGAGTGCCTGATTGGGGAACACCTGCAGGCTGGG
ATGAGCACCACCTTCCTGGTGTACTCTAAGAAATGTCAGACCCCACTGGGGATGGCCTCTGGACATATCAGGGACTTC
CAGATCACAGCTTCTGGACAGTATGGACAGTGGGCTCCAAAGCTGGCTAGACTGCACTATTCTGGCTCCATCAATGCC
TGGTCTACCAAAGAGCCATTCTCCTGGATCAAGGTGGACCTGCTGGCCCCCATGATCATCCATGGAATCAAAACCCAG
GGAGCTAGGCAGAAGTTCAGCTCTCTGTACATCTCCCAGTTTATCATCATGTATAGCCTGGATGGGAAGAAATGGCAG
ACCTACAGAGGCAATTCCACTGGGACCCTGATGGTCTTCTTTGGAAATGTGGATTCCTCTGGCATCAAGCACAACATC
TTCAATCCACCCATCATTGCCAGGTACATCAGGCTGCATCCTACCCACTATAGCATCAGGTCTACCCTGAGAATGGAG
CTGATGGGATGTGACCTGAACAGCTGTTCTATGCCACTGGGCATGGAGTCCAAGGCTATCTCTGATGCCCAGATCACA
GCTTCTTCCTACTTCACCAATATGTTTGCTACCTGGTCCCCAAGCAAGGCTAGACTGCACCTGCAGGGAAGATCCAAT
GCTTGGAGACCCCAGGTGAACAATCCTAAGGAGTGGCTGCAGGTGGACTTCCAGAAAACCATGAAGGTCACAGGGGTG
ACCACCCAGGGAGTGAAATCTCTGCTGACCTCCATGTATGTCAAGGAGTTCCTGATCAGCTCTTCCCAGGATGGCCAC
CAGTGGACCCTGTTCTTTCAGAATGGCAAGGTCAAAGTGTTCCAGGGGAATCAGGACTCTTTTACCCCAGTGGTGAAC
TCCCTGGATCCTCCACTGCTGACCAGGTACCTGAGAATCCATCCTCAGAGCTGGGTGCACCAGATTGCTCTGAGAATG
GAGGTCCTGGGATGTGAAGCTCAGGACCTGTATTGA.

The CpG deleted, liver codon optimized HSQ sequence is SEQ ID NO: 2:

ATGCAGATTGAACTGTCTACCTGTTTCTTTCTGTGCCTGCTGAGGTTTTGTTTTTCTGCTACCAGAAGATACTACCTG
GGAGCTGTGGAACTGAGCTGGGATTACATGCAGTCTGACCTGGGAGAGCTGCCTGTGGATGCTAGATTCCCACCTAGA
GTCCCTAAGTCCTTCCCCTTCAACACCTCTGTGGTCTACAAGAAAACCCTGTTTGTGGAGTTTACAGACCACCTGTTC
AACATTGCTAAGCCTAGACCACCATGGATGGGACTGCTGGGACCAACCATCCAGGCAGAGGTGTATGACACAGTGGTC
ATCACCCTGAAAAACATGGCTTCTCACCCTGTGTCCCTGCATGCTGTGGGAGTCTCCTACTGGAAGGCCTCTGAAGGG
GCTGAGTATGATGATCAGACCAGCCAGAGGGAAAAAGAGGATGATAAGGTGTTCCCTGGAGGGTCCCATACCTATGTG
TGGCAGGTCCTGAAGGAGAATGGACCAATGGCTTCTGACCCTCTGTGCCTGACCTACTCTTATCTGTCCCATGTGGAC
CTGGTCAAGGATCTGAACTCTGGCCTGATTGGGGCTCTGCTGGTGTGTAGGGAAGGGTCCCTGGCCAAGGAGAAAACC
CAGACCCTGCATAAGTTCATCCTGCTGTTTGCTGTGTTTGATGAAGGAAAAAGCTGGCACTCTGAGACCAAGAACTCT
CTGATGCAGGACAGGGATGCTGCTTCTGCCAGAGCTTGGCCCAAGATGCACACAGTGAATGGCTATGTCAATAGGAGC
CTGCCTGGACTGATTGGCTGCCACAGAAAGTCTGTGTATTGGCATGTCATTGGAATGGGCACCACCCCTGAAGTGCAC
AGCATCTTCCTGGAGGGGCATACCTTTCTGGTCAGGAACCACAGGCAGGCTAGCCTGGAGATCTCTCCAATCACCTTC
CTGACAGCCCAGACCCTGCTGATGGACCTGGGACAGTTCCTGCTGTTTTGCCACATCTCCAGCCACCAGCATGATGGC
ATGGAGGCTTATGTGAAAGTGGACTCCTGTCCTGAGGAACCTCAGCTGAGGATGAAGAACAATGAGGAAGCTGAAGAC
TATGATGATGACCTGACAGACTCTGAGATGGATGTGGTCAGGTTTGATGATGATAACTCTCCCTCCTTTATCCAGATC
AGGTCTGTGGCCAAGAAACACCCTAAGACCTGGGTCCATTACATTGCTGCTGAGGAAGAGGACTGGGATTATGCTCCA
CTGGTGCTGGCCCCTGATGATAGATCCTACAAAAGCCAGTATCTGAACAATGGACCCCAGAGGATTGGCAGAAAGTAC
AAGAAAGTGAGGTTCATGGCTTATACAGATGAGACCTTTAAGACCAGAGAAGCCATCCAGCATGAGTCTGGGATCCTG

GGACCTCTGCTGTATGGGGAAGTGGGGGACACCCTGCTGATCATCTTCAAGAACCAGGCCAGCAGGCCTTACAATATC
TATCCACATGGCATCACAGATGTGAGACCTCTGTACTCCAGGAGGCTGCCAAAGGGGGTGAAACACCTGAAGGACTTC
CCAATCCTGCCTGGGGAAATCTTTAAGTATAAATGGACAGTCACAGTGGAGGATGGGCCCACCAAGTCTGACCCTAGG
TGCCTGACCAGATACTATTCTTCCTTTGTGAATATGGAGAGAGACCTGGCTTCTGGACTGATTGGACCCCTGCTGATC
TGTTACAAAGAGTCTGTGGATCAGAGGGGCAACCAGATCATGTCTGACAAGAGGAATGTGATCCTGTTCTCTGTCTTT
GATGAAAACAGGTCTTGGTACCTGACAGAGAACATCCAGAGGTTCCTGCCTAATCCAGCTGGAGTGCAGCTGGAAGAT
CCTGAGTTCCAGGCCTCTAACATCATGCATTCCATCAATGGCTATGTGTTTGACTCCCTGCAGCTGTCTGTGTGCCTG
CATGAGGTGGCTTACTGGTATATCCTGAGCATTGGAGCCCAGACAGATTTCCTGTCTGTGTTCTTTTCTGGCTACACC
TTTAAGCATAAAATGGTGTATGAGGACACCCTGACCCTGTTCCCATTTTCTGGAGAAACTGTGTTCATGAGCATGGAG
AATCCTGGGCTGTGGATCCTGGGATGCCACAACTCTGATTTCAGGAATAGAGGGATGACAGCCCTGCTGAAAGTGAGC
TCTTGTGACAAGAACACAGGAGACTACTATGAAGATAGCTATGAGGACATCTCTGCTTATCTGCTGTCCAAAAACAAT
GCCATTGAGCCCAGGAGCTTCTCTCAGAACCCTCCAGTGCTGAAGAGGCACCAGAGGGAGATCACCAGAACCACCCTG
CAGTCTGATCAGGAAGAGATTGACTATGATGATACCATCTCTGTGGAAATGAAGAAAGAGGACTTTGATATCTATGAT
GAAGATGAGAACCAGTCTCCCAGGTCCTTCCAGAAGAAAACCAGACATTACTTTATTGCTGCTGTGGAGAGGCTGTGG
GACTATGGCATGTCCAGCTCTCCTCATGTGCTGAGAAATAGAGCTCAGTCTGGATCTGTCCCACAGTTCAAGAAAGTG
GTCTTCCAGGAGTTTACAGATGGAAGCTTTACCCAGCCACTGTACAGGGGAGAACTGAATGAGCACCTGGGGCTGCTG
GGACCCTATATCAGGGCTGAAGTGGAGGATAACATCATGGTCACCTTCAGGAATCAGGCCAGCAGACCCTACTCTTTT
TATTCCAGCCTGATCTCCTATGAAGAGGACCAGAGACAGGGAGCTGAACCAAGAAAAAACTTTGTGAAGCCTAATGAG
ACCAAAACCTACTTTTGGAAGGTGCAGCACCATATGGCCCCTACCAAAGATGAGTTTGATTGCAAGGCCTGGGCTTAT
TTTTCTGATGTGGATCTGGAGAAGGATGTCCACTCTGGCCTGATTGGGCCACTGCTGGTGTGTCATACCAACACCCTG
AATCCAGCTCATGGAAGGCAGGTGACAGTCCAGGAATTTGCCCTGTTCTTTACCATCTTTGATGAGACCAAGAGCTGG
TACTTCACAGAAAACATGGAGAGGAATTGCAGAGCCCCATGTAACATCCAGATGGAAGACCCCACCTTCAAGGAGAAC
TACAGATTTCATGCTATCAATGGGTATATCATGGATACCCTGCCAGGACTGGTCATGGCTCAGGACCAGAGGATCAGA
TGGTACCTGCTGAGCATGGGGTCTAATGAGAATATCCACTCCATCCATTTCTCTGGACATGTGTTTACAGTAAGGAAG
AAAGAAGAGTACAAGATGGCCCTGTACAACCTGTATCCTGGGGTGTTTGAAACAGTGGAGATGCTGCCTTCCAAGGCT
GGGATCTGGAGGGTGGAATGCCTGATTGGGGAGCACCTGCATGCTGGAATGTCTACCCTGTTCCTGGTGTACTCCAAT
AAGTGTCAGACCCCCCTGGGGATGGCTTCTGGACATATCAGGGACTTCCAGATCACAGCTTCTGGACAGTATGGACAG
TGGGCTCCTAAGCTGGCTAGACTGCACTATTCTGGCTCCATCAATGCTTGGTCTACCAAAGAGCCTTTCTCCTGGATC
AAGGTGGACCTGCTGGCTCCAATGATCATCCATGGCATCAAAACCCAGGGGGCCAGGCAGAAGTTCTCTTCCCTGTAC
ATCAGCCAGTTTATCATCATGTATTCTCTGGATGGGAAGAAATGGCAGACCTACAGAGGCAATTCCACAGGGACCCTG
ATGGTGTTCTTTGGCAATGTGGACAGCTCTGGGATCAAGCACAACATCTTCAATCCCCCTATCATTGCCAGGTACATC
AGACTGCACCCAACCCATTATTCCATCAGGAGCACCCTGAGAATGGAGCTGATGGGGTGTGATCTGAACAGCTGTTCT
ATGCCCCTGGGAATGGAGTCTAAGGCCATCTCTGATGCTCAGATCACAGCCTCCAGCTACTTCACCAATATGTTTGCT
ACCTGGTCCCCAAGCAAGGCTAGACTGCATCTGCAGGGAAGAAGCAATGCTTGGAGACCACAGGTGAACAATCCCAAG
GAGTGGCTGCAGGTGGACTTCCAGAAAACCATGAAGGTGACAGGAGTCACCACCCAGGGAGTGAAAAGCCTGCTGACC
TCTATGTATGTCAAGGAGTTCCTGATCTCTTCCAGCCAGGATGGGCACCAGTGGACCCTGTTCTTTCAGAATGGAAAG
GTGAAAGTCTTCCAGGGCAATCAGGATTCCTTTACCCCTGTGGTCAACAGCCTGGACCCACCCCTGCTGACCAGGTAC
CTGAGAATCCACCCACAGTCCTGGGTGCATCAGATTGCTCTGAGGATGGAAGTCCTGGGCTGTGAGGCCCAGGACCTG
TATTGA

This approach increased the LCAI from 0.62 to 0.86 for B domain deleted human fVIII (HSQ). *In vitro* expression of the optimized fVIII sequences was assessed in HepG2 cells transiently transfected with corresponding fVIII expression plasmids. The codon-optimization in the nucleic acid sequence coding for HSQ increased expression of HSQ 7.4 fold, and expressed as efficiently as non-codon optimized ET3 (FIG. 3A). The codon-optimization in the nucleic acid sequence coding for HSQ increased expression of ET3 5.6 fold. When tested *in vivo* by hydrodynamic injection of plasmid carrying the codon optimized fVIII variants into mice, HSQ activity rose to levels that could be detected by the assay which were nearly equivalent to the fVIII levels seen in the non-codon optimized ET3 (FIG. 3B). Further, a 17 fold increase in activity was observed for the codon optimized ET3 (FIG. 3B).

Translation efficacy can sometimes also be improved through optimization of GC content, mRNA secondary structure, premature PolyA sites, RNA instability motif, stable free energy of mRNA, internal chi sites, ribosomal binding sites, cryptic splicing sites, negative CpG islands, SD sequence, TATA boxes, and cyptic terminal signals, etc. FIG. 4 illustrates changes that were made to an AAV cassette encoding the ET3 codon-optimized CpG deleted sequence to increase its production. Several cis-acting elements

within the HSQ sequence were additional modified to increase fVIII expression, as summarized in the following table.

CIS-Acting Elements	Optimized	Original
Splice(GGTAAG)	0	2
Splice(GGTGAT)	0	1
PolyA(AATAAA) (4789 - AATAAA)	1	1
PolyA(ATTAAA)	0	0
Destabilizing(ATTTA)	0	6
PolyT(TTTTTT) (4827 - TTTTTT)	1	2
PolyA(AAAAAAA)	0	1

In addition to the liver-optimized codon sequences provided above, the encoding sequence for the ET3 and HSQ fVIII proteins were codon-optimized for expression in myeloid cells. Using the sequences of highly expressed genes in the human myeloid cells, a custom codon-usage bias table specific for the human liver was constructed (see FIG. 2C) that differs substantially from the most prevalent codons used in total human coding sequences (FIG. 2B). Optimized coding sequence of the ET3 and HSQ variants of fVIII was developed with the most statistically prevalent codons identified by the liver-usage bias analysis. In addition, CpG DNA motifs in the myeloid-codon optimized coding sequence for ET3 and HSQ were removed because they may lead to gene methylation and silencing.

The CpG deleted, myeloid codon optimized ET3 sequence is SEQ ID NO: 125:

ATGCAGCTGGAGCTCTCAACCTGTGTGTTCCTCTGCCTGCTCCCCCTGGGATTTTCAGCTATCAGGAGATAC
TATCTGGGAGCAGTGGAACTGTCCTGGGACTACAGGCAGTCAGAGCTGCTCAGAGAACTGCATGTGGATACTAGGTTC
CCTGCAACAGCTCCTGGAGCACTGCCACTGGGACCTTCAGTGCTGTACAAGAAAACTGTCTTTGTGGAGTTTACAGAC
CAGCTGTTCAGTGTGGCCAGGCCCAGGCCCCCCTGGATGGGGCTGCTGGGACCCACCATCCAGGCTGAAGTGTATGAT
ACTGTGGTGGTGACCCTGAAAAACATGGCCTCTCATCCAGTCAGCCTGCATGCTGTGGGAGTGAGCTTCTGGAAGAGC
AGTGAGGGAGCTGAGTATGAAGACCATACCTCACAGAGGGAGAAAGAAGATGATAAGGTGCTGCCAGGAAAAAGCCAG
ACCTATGTGTGGCAGGTGCTGAAGGAGAATGGCCCTACAGCTTCAGATCCTCCCTGCCTCACATACTCTTATCTGAGC
CATGTGGATCTGGTGAAGGACCTCAATAGTGGCCTGATTGGGGCACTGCTGGTGTGCAGAGAGGGGTCCCTCACAAGG
GAAAGAACTCAGAACCTGCATGAGTTTGTCCTGCTCTTTGCTGTGTTTGATGAGGGAAAGTCCTGGCACTCAGCAAGG
AATGACAGCTGGACCAGGGCTATGGACCCAGCACCAGCCAGAGCTCAGCCAGCTATGCACACTGTCAATGGCTATGTG
AATAGGTCCCTGCCTGGACTCATTGGCTGCCATAAGAAATCAGTCTATTGGCATGTGATTGGAATGGGCACCAGCCCA
GAGGTGCATTCCATCTTCCTGGAAGGCCACACATTTCTGGTCAGGCACCATAGACAGGCCAGCCTGGAGATCAGCCCA
CTGACTTTCCTCACAGCACAGACATTTCTGATGGACCTGGGGCAGTTCCTGCTCTTTTGCCATATCTCAAGTCACCAT
CATGGAGGGATGGAGGCTCATGTCAGGGTGGAAAGCTGTGCAGAGGAACCTCAGCTGAGGAGGAAGGCAGATGAGGAA
GAGGACTATGATGATAACCTGTATGACTCAGATATGGATGTGGTGAGGCTGGATGGAGATGATGTCAGCCCATTCATC
CAGATCAGGTCAGTGGCTAAGAAACACCCTAAGACCTGGGTCCACTACATTGCAGCTGAAGAGGAAGATTGGGACTAT
GCACCCCTGGTGCTGGCCCCAGATGATAGAAGTTACAAATCTCAGTATCTGAACAATGGGCCCCAGAGGATTGGAAGG
AAGTACAAGAAAGTGAGGTTCATGGCTTATACTGATGAGACCTTTAAGACAAGAGAGGCAATCCAGCATGAAAGTGGC
ATCCTGGGACCACTGCTCTATGGAGAAGTGGGGGATACCCTGCTCATCATCTTCAAGAACCAGGCCTCAAGGCCTTAC
AATATCTATCCCCATGGCATCACAGATGTGAGGCCTCTCTACAGCAGGAGACTGCCCAAGGGAGTCAAACACCTCAAG
GATTTCCCCATCCTGCCAGGGGAAATCTTCAAGTATAAATGGACAGTCACTGTGGAAGATGGGCCAACTAAGTCAGAT
CCTAGGTGCCTGACCAGGTACTATTCTAGCTTTGTGAACATGGAGAGGGACCTGGCTTCAGGACTGATTGGACCTCTG
CTCATCTGCTACAAAGAATCAGTGGACCAGAGGGGCAACCAGATCATGAGTGATAAGAGAAATGTCATCCTGTTCTCA
GTGTTTGATGAGAATAGGAGTTGGTATCTGACAGAAAACATCCAGAGGTTCCTGCCTAATCCTGCAGGAGTGCAGCTG
GAGGACCCAGAATTTCAGGCTTCAAACATCATGCATAGTATCAATGGCTATGTGTTTGATAGTCTGCAGCTCTCTGTC
TGCCTGCATGAGGTGGCCTACTGGTATATCCTCAGCATTGGAGCTCAGACTGACTTCCTGAGTGTGTTCTTTTCAGGC
TACACATTCAAGCATAAGATGGTCTATGAAGATACCCTGACACTCTTCCCCTTTTCTGGGGAGACTGTGTTTATGAGC
ATGGAAAACCCAGGCCTGTGGATTCTGGGGTGCCACAACAGTGACTTCAGGAATAGAGGGATGACTGCTCTGCTCAAA
GTGTCCTCATGTGATAAGAATACTGGAGATTACTATGAGGACTCTTATGAAGATATCAGTGCATATCTGCTCTCCAAA
AACAATGCCATTGAGCCCAGGTCATTTGCTCAGAACAGTAGACCACCTTCTGCAAGTGCACCAAAGCCTCCAGTGCTG
AGGAGACACCAGAGGGACATCAGCCTGCCAACCTTCCAGCCTGAGGAAGATAAAATGGACTATGATGATATCTTCTCC
ACTGAGACCAAGGGGGAAGATTTTGACATCTATGGAGAGGATGAAAACCAGGACCCCAGGTCCTTCCAGAAGAGGACC
AGACACTACTTTATTGCAGCTGTGGAGCAGCTGTGGGACTATGGCATGTCTGAATCACCTAGAGCTCTGAGGAACAGA
GCACAGAATGGGGAGGTGCCCAGGTTCAAGAAAGTGGTGTTCAGAGAATTTGCAGATGGCTCTTTTACCCAGCCTAGC
TACAGGGGGGAGCTCAACAAGCATCTGGGGCTGCTGGGACCCTATATCAGAGCAGAGGTGGAAGATAACATCATGGTG
ACATTCAAGAATCAGGCCTCAAGACCCTACAGTTTTTATAGTTCTCTGATCAGCTACCCAGATGATCAGGAGCAGGGG

GCTGAACCAAGGCACAACTTTGTGCAGCCTAATGAGACAAGAACTTACTTTTGGAAGGTCCAGCATCACATGGCTCCC
ACAGAGGATGAGTTTGACTGCAAGGCCTGGGCATATTTTTCTGATGTGGACCTGGAGAAGGATGTGCATAGTGGCCTC
ATTGGGCCACTGCTCATCTGCAGGGCAAACACACTGAATGCTGCACATGGCAGGCAGGTCACTGTGCAGGAGTTTGCC
CTGTTCTTTACAATCTTTGATGAAACTAAGTCCTGGTACTTCACAGAGAATGTGGAAAGGAATTGCAGAGCCCCCTGC
CATCTCCAGATGGAGGACCCAACTCTGAAGGAAAACTACAGGTTCCATGCTATCAATGGATATGTCATGGATACACTG
CCAGGCCTGGTGATGGCACAGAACCAGAGGATCAGGTGGTATCTGCTCAGCATGGGGTCCAATGAGAATATCCATTCT
ATCCACTTCTCAGGACATGTCTTTTCAGTGAGGAAGAAAGAGGAATATAAAATGGCTGTGTACAATCTGTATCCAGGG
GTCTTTGAGACAGTGGAAATGCTGCCTAGCAAAGTGGGGATCTGGAGAATTGAGTGCCTCATTGGAGAACACCTGCAG
GCAGGGATGTCCACCACATTTCTGGTGTACTCAAAGAAATGCCAGACTCCCCTGGGGATGGCAAGTGGACATATCAGG
GACTTCCAGATCACTGCATCAGGACAGTATGGACAGTGGGCACCAAAGCTGGCTAGGCTCCACTATAGTGGCTCTATC
AATGCTTGGAGTACCAAAGAGCCTTTCTCTTGGATCAAGGTGGATCTGCTGGCCCCCATGATCATCCATGGAATCAAA
ACACAGGGAGCTAGACAGAAGTTCAGCTCCCTGTACATCAGTCAGTTTATCATCATGTATTCTCTGGATGGGAAGAAA
TGGCAGACCTACAGGGGCAATAGCACTGGGACACTGATGGTCTTCTTTGGAAATGTGGATTCAAGTGGCATCAAGCAC
AACATCTTCAATCCTCCCATCATTGCCAGGTACATCAGACTGCATCCCACACACTATTCAATCAGGAGTACTCTCAGA
ATGGAGCTGATGGGGTGTGACCTCAACAGCTGCTCCATGCCACTGGGAATGGAATCCAAGGCAATCTCAGATGCCCAG
ATCACTGCTTCTAGCTACTTCACCAATATGTTTGCAACATGGTCACCCAGTAAAGCAAGGCTGCACCTCCAGGGAAGG
TCCAATGCTTGGAGACCCCAGGTGAACAATCCAAAGGAGTGGCTGCAGGTGGACTTTCAGAAAACCATGAAGGTCACA
GGGGTGACTACCCAGGGAGTGAAAAGTCTGCTCACCTCTATGTATGTCAAGGAGTTCCTGATCTCCTCAAGTCAGGAT
GGCCACCAGTGGACACTGTTCTTTCAGAATGGCAAGGTCAAAGTGTTCCAGGGGAATCAGGACAGCTTTACACCAGTG
GTGAACAGCCTGGACCCCCCTCTGCTCACTAGATATCTGAGAATCCATCCACAGAGCTGGGTGCACCAGATTGCACTC
AGAATGGAGGTCCTGGGCTGTGAAGCCCAGGACCTGTATTGA

The CpG deleted, myeloid codon optimized HSQ sequence is SEQ ID NO: 126:

ATGCAGATTGAGCTCAGCACCTGCTTCTTTCTGTGCCTGCTCAGGTTCTGCTTTTCAGCCACAAGGAGATACTATCTG
GGAGCTGTGGAACTGTCATGGGATTACATGCAGAGTGACCTGGGAGAGCTCCCTGTGGATGCTAGGTTCCCCCCAAGG
GTCCCAAAGTCTTTCCCTTTTAATACCAGTGTGGTCTATAAGAAAACACTCTTTGTGGAATTTACTGATCACCTGTTC
AACATTGCAAAGCCAAGGCCTCCCTGGATGGGACTGCTGGGACCTACCATCCAGGCTGAGGTGTATGACACTGTGGTC
ATCACACTGAAAAACATGGCATCTCACCCTGTCAGCCTGCATGCAGTGGGAGTCAGCTACTGGAAGGCTTCAGAAGGG
GCAGAGTATGATGATCAGACAAGCCAGAGAGAAAAAGAGGATGATAAGGTGTTCCCAGGAGGGAGCCATACTTATGTG
TGGCAGGTCCTGAAGGAGAATGGCCCAATGGCCAGTGACCCACTGTGCCTCACCTACTCATATCTGAGTCATGTGGAC
CTGGTCAAGGATCTCAACTCAGGCCTGATTGGGGCACTGCTGGTGTGCAGGGAAGGCTCACTGGCCAAGGAGAAAACC
CAGACACTGCATAAGTTCATCCTGCTCTTTGCTGTGTTTGATGAAGGGAAATCTTGGCACAGTGAGACCAAGAACAGT
CTGATGCAGGACAGGGATGCTGCTTCTGCCAGAGCTTGGCCCAAGATGCACACAGTGAATGGATATGTCAATAGGTCC
CTGCCAGGACTCATTGGCTGCCACAGAAAGTCAGTGTATTGGCATGTCATTGGAATGGGCACCACACCAGAAGTGCAC
AGCATCTTCCTGGAGGGGCATACCTTTCTGGTCAGGAACCACAGGCAGGCCAGCCTGGAGATCAGCCCAATCACCTTC
CTGACAGCCCAGACTCTGCTCATGGATCTGGGGCAGTTCCTGCTCTTTTGCCACATCAGCTCCCACCAGCATGATGGA
ATGGAGGCATATGTGAAAGTGGACTCCTGCCCAGAGGAACCACAGCTGAGGATGAAGAACAATGAGGAAGCTGAAGAC
TATGATGATGACCTGACAGACTCAGAGATGGATGTGGTCAGGTTTGATGATGATAACAGCCCCTCCTTTATCCAGATC
AGAAGTGTGGCCAAGAAACACCCAAAGACATGGGTCCATTACATTGCAGCTGAGGAAGAGGACTGGGATTATGCACCT
CTGGTGCTGGCCCCAGATGATAGATCCTACAAATCACAGTATCTGAACAATGGACCCCAGAGGATTGGCAGAAAGTAC
AAGAAAGTGAGGTTCATGGCCTATACTGATGAAACATTTAAGACTAGAGAAGCTATCCAGCATGAGTCAGGCATCCTG
GGACCACTGCTCTATGGAGAAGTGGGGGACACCCTGCTCATCATCTTCAAGAACCAGGCTTCCAGGCCATACAATATC
TATCCTCATGGCATCACAGATGTGAGACCACTCTACTCAAGGAGACTGCCTAAGGGAGTCAAACACCTCAAGGACTTC
CCTATCCTGCCAGGGGAAATCTTTAAGTATAAATGGACTGTGACAGTGGAGGATGGGCCCACTAAGAGTGACCCAAGG
TGCCTGACCAGATACTATTCAAGTTTTGTGAATATGGAAAGGGATCTGGCATCAGGACTGATTGGACCTCTGCTCATC
TGCTACAAAGAGAGTGTGGATCAGAGGGGCAACCAGATCATGTCAGACAAGAGGAATGTGATCCTGTTCAGTGTCTTT
GATGAAAACAGGTCTTGGTATCTGACAGAGAACATCCAGAGATTCCTGCCAAATCCTGCAGGGGTGCAGCTGGAAGAT
CCAGAGTTTCAGGCCTCAAACATCATGCATAGTATCAATGGATATGTGTTTGACAGTCTGCAGCTCTCTGTGTGCCTG
CATGAAGTGGCCTACTGGTATATCCTGTCCATTGGAGCTCAGACAGATTTCCTGAGTGTGTTCTTTTCAGGCTACACT
TTTAAGCATAAAATGGTCTATGAGGACACACTGACTCTCTTCCCTTTTAGTGGGGAAACAGTGTTTATGAGCATGGAG
AATCCAGGGCTGTGGATTCTGGGATGCCACAACAGTGATTTCAGGAATAGAGGCATGACTGCTCTGCTCAAAGTGTCT
AGCTGTGACAAGAACACAGGGGACTACTATGAAGATTCTTATGAGGACATCAGTGCTTATCTGCTCTCCAAAAACAAT
GCAATTGAACCCAGATCATTCAGTCAGAATCCACCTGTGCTGAAGAGGCACCAGAGAGAGATCACTAGGACTACCCTG
CAGTCAGATCAGGAAGAGATTGACTATGATGATACCATCTCAGTGGAAATGAAGAAAGAGGACTTTGATATCTATGAT
GAAGATGAGAACCAGAGTCCAAGGTCTTTCCAGAAGAAAACCAGACATTACTTTATTGCTGCAGTGGAGAGGCTGTGG
GATTATGGAATGTCCTCAAGTCCACATGTGCTGAGGAATAGGGCACAGTCTGGCAGTGTCCCTCAGTTCAAGAAAGTG
GTCTTCCAGGAGTTTACAGATGGCAGCTTCACTCAGCCTCTGTACAGGGGAGAACTCAATGAGCACCTGGGGCTGCTG
GGACCCTATATCAGAGCTGAAGTGGAGGATAACATCATGGTCACCTTCAGGAATCAGGCTTCAAGACCCTACAGTTTT
TATTCTAGCCTGATCAGCTATGAAGAGGACCAGAGGCAGGGAGCTGAACCTAGGAAAAACTTTGTGAAGCCAAATGAG
ACCAAAACATACTTTTGGAAGGTCCAGCACCACATGGCACCAACCAAAGATGAGTTTGATTGCAAGGCATGGGCCTAT
TTTTCAGATGTGGATCTGGAGAAGGATGTCCACAGTGGCCTCATTGGGCCTCTGCTGGTGTGCCATACTAACACCCTG
AATCCAGCTCATGGCAGGCAGGTGACAGTCCAGGAGTTTGCACTGTTCTTTACCATCTTTGATGAGACAAAGTCCTGG
TACTTCACTGAAAACATGGAGAGGAATTGCAGAGCTCCTTGCAACATCCAGATGGAAGACCCCACCTTCAAGGAGAAC

TACAGATTTCATGCAATCAATGGGTATATCATGGATACACTGCCAGGACTGGTGATGGCCCAGGACCAGAGGATCAGA
TGGTATCTGCTCAGCATGGGGTCCAATGAGAATATCCACTCTATCCATTTCAGTGGACATGTGTTTACAGTCAGAAAG
AAAGAAGAGTATAAAATGGCCCTGTACAACCTCTATCCAGGAGTGTTTGAAACAGTGGAGATGCTGCCAAGCAAGGCT
GGGATCTGGAGGGTGGAATGCCTCATTGGGGAGCACCTGCATGCAGGAATGTCAACCCTGTTTCTGGTCTACAGTAAT
AAGTGCCAGACACCTCTGGGAATGGCAAGTGGACATATCAGGGATTTCCAGATCACTGCTAGTGGACAGTATGGACAG
TGGGCACCAAAGCTGGCTAGACTCCACTATTCAGGCTCAATCAATGCTTGGTCCACCAAAGAGCCATTCTCATGGATC
AAGGTGGACCTGCTGGCTCCTATGATCATCCATGGCATCAAAACACAGGGGGCAAGGCAGAAGTTCTCCTCACTGTAC
ATCTCTCAGTTTATCATCATGTATAGCCTGGATGGCAAGAAATGGCAGACCTACAGGGGCAATAGCACAGGGACTCTG
ATGGTGTTCTTTGGCAATGTGGACAGCAGTGGGATCAAGCACAACATCTTCAATCCCCCAATCATTGCAAGGTACATC
AGACTGCACCCCACCCATTATTCAATCAGGAGTACACTCAGGATGGAACTGATGGGGTGTGATCTCAACAGTTGCTCT
ATGCCACTGGGAATGGAGTCCAAGGCAATCTCAGATGCCCAGATCACTGCTAGCTCCTACTTCACTAATATGTTTGCT
ACCTGGAGCCCCTCCAAAGCAAGGCTGCACCTCCAGGGAAGGAGCAATGCATGGAGGCCTCAGGTGAACAATCCCAAG
GAATGGCTGCAGGTGGATTTCCAGAAAACTATGAAGGTGACTGGAGTCACAACTCAGGGAGTGAAAAGTCTGCTCACT
TCTATGTATGTCAAGGAGTTCCTGATCTCAAGTTCTCAGGATGGCCACCAGTGGACCCTGTTCTTTCAGAATGGAAAG
GTGAAAGTCTTCCAGGGCAATCAGGATTCCTTTACACCAGTGGTCAACTCACTGGACCCTCCCCTGCTCACTAGATAT
CTGAGAATCCACCCTCAGAGCTGGGTGCATCAGATTGCTCTCAGAATGGAAGTCCTGGGCTGTGAGGCACAGGACCTG
TATTGA

In vitro expression of the non-optimized, liver-optimized, and myeloid optimized fVIII sequences was assessed in HepG2 cells transiently transfected with corresponding fVIII expression plasmids (FIG.5). The tissue specific optimization lead to increased FVIII activity in the HepG2 (hepatic) cells expressing the liver-optimized fVIII compared to either non-optimized or myeloid optimized forms of ET3 or HSQ. These results show that liver optimization specifically benefits expression in hepatocyte derived cells and that expression of myeloid-optimized FVIII does not benefit expression in HepG2 cells.

Further, when these experiments were repeated using non-human cells (baby hamster kidney cells), it was found that the human-specific sequence optimization led to decreased expression in the non-human cells (FIG. 6).

Factor IX

Similar to the codon-optimization for clotting fVIII discussed above, fIX sequences were also codon optimized for expression in hepatocytes using the same codon optimization tables used for clotting factor fVIII. The fIX sequences selected for optimization include fIX with the pro-thrombogenic "Padua" mutation R338L (" fIX Padua," see Paolo et al, "X-Linked Thrombophilia with a Mutant Factor IX" N Engl J Med; 361:1671-1675, 2009) and fIX with the thrombophelic "Malmo" variant 148T (fIX Malmo," see Graham et al, "The Malmo Polymorphism of Coagulation Factor IX, An Immunologic Polymorphism Due to Dimorphism of Residue 148 That Is in Linkage Disequilibrium with Two Other FIX Polymorphisms," Am. J. Hum. Genet. 42:573-580, 1988)

Liver and myeloid optimized fIX cDNAs were designed and optimized according to the liver and myeloid tables shown in FIG. 2. All instances of "cg" in their sequence removed through synonymous codon substitution. Both cDNAs incorporate the Padua and Malmo substitutions.

Liver codon optimized fIX with Padua/Malmo mutations and no CpG (Version 1) (SEQ ID NO: 124)

ATGCAGAGGGTCAATATGATCATGGCTGAATCTCCTGGGCTGATCACCATTTGCCTGCTGGGATACCTGCTGTCTGCT
GAGTGTACAGTGTTCCTGGACCATGAGAATGCCAATAAGATCCTGAACAGGCCCAAAAGATACAATTCTGGAAAGCTG
GAGGAATTTGTGCAGGGCAACCTGGAGAGGGAATGCATGGAGGAAAAGTGTAGCTTTGAGGAAGCTAGGGAGGTGTTT
GAAAACACAGAGAGGACCACAGAATTCTGGAAGCAGTATGTGGATGGAGATCAGTGTGAGTCCAACCCCTGTCTGAAT
GGAGGGTCTTGCAAAGATGATATCAACTCCTATGAGTGCTGGTGTCCTTTTGGATTTGAAGGCAAAAATTGTGAGCTG
GATGTGACCTGTAACATCAAGAATGGCAGGTGTGAGCAGTTCTGTAAAAACTCTGCTGATAATAAGGTGGTCTGCAGC

TGTACAGAAGGCTATAGGCTGGCTGAGAACCAGAAGAGCTGTGAACCAGCTGTGCCCTTCCCTTGTGGGAGGGTGTCT
GTCAGCCAGACCTCTAAGCTGACCAGAGCTGAGACTGTGTTCCCAGATGTGGATTATGTCAACTCCACAGAGGCTGAA
ACCATCCTGGACAACATCACCCAGTCTACCCAGTCCTTCAATGACTTTACCAGAGTGGTGGGAGGAGAGGATGCCAAA
CCAGGCCAGTTCCCCTGGCAGGTGGTCCTGAATGGGAAGGTGGATGCTTTTTGTGGGGGATCCATTGTGAATGAGAAA
TGGATTGTCACAGCTGCTCACTGTGTGGAGACAGGGGTCAAGATCACTGTGGTGGCTGGAGAGCACAACATTGAGGAA
ACAGAACATACTGAGCAGAAGAGGAATGTGATCAGAATCATCCCTCACCATAACTACAATGCTGCTATCAACAAATAT
AATCATGACATTGCCCTGCTGGAACTGGATGAGCCTCTGGTGCTGAACAGCTATGTCACCCCAATCTGCATTGCTGAC
AAAGAGTATACCAATATCTTCCTGAAGTTTGGATCTGGATATGTGTCTGGATGGGGAAGGGTCTTCCACAAGGGCAGG
TCTGCCCTGGTGCTGCAGTATCTGAGGGTGCCTCTGGTGGACAGAGCTACCTGCCTGCTGTCTACCAAGTTCACCATC
TACAACAATATGTTCTGTGCTGGATTTCATGAGGGAGGCAGGGACTCCTGTCAGGGGGATTCTGGAGGCCCACATGTG
ACAGAGGTGGAAGGCACCAGCTTCCTGACTGGCATCATCTCTTGGGGGGAGGAATGTGCTATGAAGGGGAAATATGGA
ATCTACACCAAAGTGAGCAGGTATGTGAACTGGATCAAAGAGAAGACCAAACTGACCTGA

Liver-Codon Optimized fIX with no CpG encoding A582 modifications (SEQ ID NO: 8)

ATGCAGAGGGTGAACATGATCATGGCTGAGTCTCCTGGACTGATCACCATCTGCCTGCTGGGCTATCTGCTGTCTGCT
GAGTGTACAGTGTTCCTGGACCATGAAAATGCTAATAAAATCCTGAACAGGCCAAAGAGGTACAATTCTGGGAAACTG
GAGGAATTTGTGCAGGGAAACCTGGAGAGGGAATGCATGGAGGAAAAGTGTAGCTTTGAGGAAGCCAGGGAGGTGTTT
GAAAATACAGAGAGGACCACAGAGTTCTGGAAACAGTATGTGGATGGGGATCAGTGTGAGTCCAACCCCTGTCTGAAT
GGAGGGTCTTGCAAGGATGATATCAACTCCTATGAGTGCTGGTGTCCTTTTGGATTTGAAGGCAAGAATTGTGAGCTG
GATGTGACCTGTAACATCAAAAATGGGAGGTGTGAGCAGTTCTGTAAGAACTCTGCTGATAATAAAGTGGTCTGCAGC
TGTACAGAAGGCTACAGGCTGGCTGAGAACCAGAAGAGCTGTGAACCAGCTGTGCCCTTCCCTTGTGGGAGGGTGTCT
GTCAGCCAGACCAGCAAGCTGACCAGAGCTGAGGCTGTGTTTCCTGATGTGGATTATGTCAACTCTACAGAGGCTGAA
ACCATCCTGGACAACATCACCCAGTCTACCCAGTCCTTCAATGACTTTACCAGGGTGGTGGGAGGGGAGGATGCTAAG
CCAGGACAGTTCCCCTGGCAGGTGGTCCTGAATGGCAAAGTGGATGCTTTTTGTGGGGGCTCCATTGTGAATGAGAAG
TGGATTGTCACAGCTGCTCACTGTGTGGAAACTGGGGTCAAGATCACAGTGGTGGCTGGAGAGCACAACATTGAGGAA
ACTGAACATACAGAGCAGAAAAGGAATGTGATCAGAATCATCCCCCACCATAACTACAATGCTGCTATCAACAAGTAT
AATCATGACATTGCCCTGCTGGAACTGGATGAGCCTCTGGTGCTGAACAGCTATGTCACCCCAATCTGCATTGCTGAC
AAGGAGTATACCAATATCTTCCTGAAATTTGGGTCTGGATATGTGTCTGGGTGGGGAAGGGTCTTCCACAAGGGAAGG
TCTGCTCTGGTGCTGCAGTATCTGAGGGTGCCCCTGGTGGACAGAGCTACCTGCCTGAGGAGCACCAAGTTCACCATC
TACAACAATATGTTCTGTGCTGGATTTCATGAGGGAGGGAGGGACTCCTGTCAGGGAGATTCTGGAGGCCCTCATGTG
ACAGAGGTGGAAGGCACCAGCTTCCTGACTGGCATCATCTCTTGGGGGGAGGAATGTGCTATGAAGGGGAAATATGGA
ATCTATACCAAGGTGTCCAGATATGTCAACTGGATCAAGGAGAAAACCAAGCTGACCTGA

Liver codon optimized fIX with no CpG including Padua and A582 modifications (SEQ ID NO: 9)

ATGCAGAGGGTGAACATGATCATGGCTGAGTCTCCTGGACTGATCACCATCTGCCTGCTGGGCTATCTGCTGTCTGCT
GAGTGTACAGTGTTCCTGGACCATGAAAATGCTAATAAAATCCTGAACAGGCCAAAGAGGTACAATTCTGGGAAACTG
GAGGAATTTGTGCAGGGAAACCTGGAGAGGGAATGCATGGAGGAAAAGTGTAGCTTTGAGGAAGCCAGGGAGGTGTTT
GAAAATACAGAGAGGACCACAGAGTTCTGGAAACAGTATGTGGATGGGGATCAGTGTGAGTCCAACCCCTGTCTGAAT
GGAGGGTCTTGCAAGGATGATATCAACTCCTATGAGTGCTGGTGTCCTTTTGGATTTGAAGGCAAGAATTGTGAGCTG
GATGTGACCTGTAACATCAAAAATGGGAGGTGTGAGCAGTTCTGTAAGAACTCTGCTGATAATAAAGTGGTCTGCAGC
TGTACAGAAGGCTACAGGCTGGCTGAGAACCAGAAGAGCTGTGAACCAGCTGTGCCCTTCCCTTGTGGGAGGGTGTCT
GTCAGCCAGACCAGCAAGCTGACCAGAGCTGAGGCTGTGTTTCCTGATGTGGATTATGTCAACTCTACAGAGGCTGAA
ACCATCCTGGACAACATCACCCAGTCTACCCAGTCCTTCAATGACTTTACCAGGGTGGTGGGAGGGGAGGATGCTAAG
CCAGGACAGTTCCCCTGGCAGGTGGTCCTGAATGGCAAAGTGGATGCTTTTTGTGGGGGCTCCATTGTGAATGAGAAG
TGGATTGTCACAGCTGCTCACTGTGTGGAAACTGGGGTCAAGATCACAGTGGTGGCTGGAGAGCACAACATTGAGGAA
ACTGAACATACAGAGCAGAAAAGGAATGTGATCAGAATCATCCCCCACCATAACTACAATGCTGCTATCAACAAGTAT
AATCATGACATTGCCCTGCTGGAACTGGATGAGCCTCTGGTGCTGAACAGCTATGTCACCCCAATCTGCATTGCTGAC
AAGGAGTATACCAATATCTTCCTGAAATTTGGGTCTGGATATGTGTCTGGGTGGGGAAGGGTCTTCCACAAGGGAAGG
TCTGCTCTGGTGCTGCAGTATCTGAGGGTGCCCCTGGTGGACAGAGCTACCTGCCTGCTGAGCACCAAGTTCACCATC
TACAACAATATGTTCTGTGCTGGATTTCATGAGGGAGGGAGGGACTCCTGTCAGGGAGATTCTGGAGGCCCTCATGTG
ACAGAGGTGGAAGGCACCAGCTTCCTGACTGGCATCATCTCTTGGGGGGAGGAATGTGCTATGAAGGGGAAATATGGA
ATCTATACCAAGGTGTCCAGATATGTCAACTGGATCAAGGAGAAAACCAAGCTGACCTGA

In addition, two other variants were constructed. The first is a liver optimized, Padua, 148T variant that is very similar to the above liver optimized sequence, except it was synthesized using an alternate version of the codon optimization algorithm.

Liver codon optimized fIX with Padua/Malmo mutations and no CpG (version 2) (SEQ ID NO: 10)

ATGCAGAGGGTGAACATGATCATGGCTGAGTCTCCTGGACTGATCACCATCTGCCTGCTGGGCTATCTGCTGTCTGCT
GAGTGTACAGTGTTCCTGGACCATGAAAATGCTAATAAAATCCTGAACAGGCCAAAGAGGTACAATTCTGGGAAACTG
GAGGAATTTGTGCAGGGAAACCTGGAGAGGGAATGCATGGAGGAAAAGTGTAGCTTTGAGGAAGCCAGGGAGGTGTTT
GAAAATACAGAGAGGACCACAGAGTTCTGGAAACAGTATGTGGATGGGGATCAGTGTGAGTCCAACCCCTGTCTGAAT
GGAGGGTCTTGCAAGGATGATATCAACTCCTATGAGTGCTGGTGTCCTTTTGGATTTGAAGGCAAGAATTGTGAGCTG
GATGTGACCTGTAACATCAAAAATGGGAGGTGTGAGCAGTTCTGTAAGAACTCTGCTGATAATAAAGTGGTCTGCAGC
TGTACAGAAGGCTACAGGCTGGCTGAGAACCAGAAGAGCTGTGAACCAGCTGTGCCCTTCCCTTGTGGGAGGGTGTCT
GTCAGCCAGACCAGCAAGCTGACCAGAGCTGAGACAGTGTTTCCTGATGTGGATTATGTCAACTCTACAGAGGCTGAA
ACCATCCTGGACAACATCACCCAGTCTACCCAGTCCTTCAATGACTTTACCAGGGTGGTGGGAGGGGAGGATGCTAAG
CCAGGACAGTTCCCCTGGCAGGTGGTCCTGAATGGCAAAGTGGATGCTTTTTGTGGGGGCTCCATTGTGAATGAGAAG
TGGATTGTCACAGCTGCTCACTGTGTGGAAACTGGGGTCAAGATCACAGTGGTGGCTGGAGAGCACAACATTGAGGAA
ACTGAACATACAGAGCAGAAAAGGAATGTGATCAGAATCATCCCCCACCATAACTACAATGCTGCTATCAACAAGTAT
AATCATGACATTGCCCTGCTGGAACTGGATGAGCCTCTGGTGCTGAACAGCTATGTCACCCCAATCTGCATTGCTGAC
AAGGAGTATACCAATATCTTCCTGAAATTTGGGTCTGGATATGTGTCTGGGTGGGGAAGGGTCTTCCACAAGGGAAGG
TCTGCTCTGGTGCTGCAGTATCTGAGGGTGCCCCTGGTGGACAGAGCTACCTGCCTGCTGAGCACCAAGTTCACCATC
TACAACAATATGTTCTGTGCTGGATTTCATGAGGGAGGGAGGGACTCCTGTCAGGGAGATTCTGGAGGCCCTCATGTG
ACAGAGGTGGAAGGCACCAGCTTCCTGACTGGCATCATCTCTTGGGGGGAGGAATGTGCTATGAAGGGGAAATATGGA
ATCTATACCAAGGTGTCCAGATATGTCAACTGGATCAAGGAGAAAACCAAGCTGACCTGA

In addition, a fIX sequence with Padua/Malmo mutations and no CpG was optimized according to the standard human codon optimization table (see FIG. 2B).

Human codon optimized fIX with Padua/Malmo mutations and no CpG (SEQ ID NO: 127)

ATGCAGAGGGTGAATATGATTATGGCTGAGTCCCCTGGGCTGATTACCATTTGCCTGCTGGGATACCTGCTGTCTGCT
GAGTGTACAGTGTTCCTGGACCATGAGAATGCAAATAAGATCCTGAACAGGCCCAAAAGATATAATAGTGGAAAGCTG
GAGGAATTTGTGCAGGGCAACCTGGAGAGAGAATGCATGGAGGAAAAGTGTAGCTTTGAGGAAGCCAGGGAGGTGTTT
GAAAATACAGAGAGAACCACAGAATTCTGGAAGCAGTATGTGGATGGAGATCAGTGTGAGAGCAACCCCTGTCTGAAT
GGAGGGAGTTGCAAAGATGATATCAACTCATATGAATGCTGGTGTCCTTTTGGATTTGAAGGCAAAAATTGTGAGCTG
GATGTGACCTGTAACATTAAGAATGGGAGGTGTGAGCAGTTTTGTAAAAACTCTGCTGATAATAAGGTGGTCTGCAGT
TGTACAGAAGGGTATAGACTGGCTGAGAACCAGAAGTCCTGTGAACCAGCTGTGCCCTTCCCTTGTGGAAGGGTGTCT
GTCTCCCAGACTTCAAAACTGACCAGAGCTGAGACTGTGTTTCCTGATGTGGATTATGTCAACAGCACAGAGGCTGAA
ACTATCCTGGACAACATTACTCAGTCTACCCAGAGTTTCAATGACTTTACCAGAGTGGTGGGAGGAGAGGATGCTAAA
CCAGGCCAGTTCCCCTGGCAGGTGGTCCTGAATGGGAAGGTGGATGCATTTTGTGGGGGATCTATTGTGAATGAGAAA
TGGATTGTCACAGCTGCTCACTGTGTGGAAACTGGGGTCAAGATCACAGTGGTGGCTGGAGAGCACAACATTGAGGAA
ACAGAACATACTGAGCAGAAGAGGAATGTGATCAGAATCATTCCTCACCATAACTACAATGCAGCCATCAACAAATAT
AATCATGACATTGCCCTGCTGGAACTGGATGAGCCTCTGGTGCTGAACAGCTATGTCACACCAATCTGCATTGCTGAC
AAGGAGTACACTAACATCTTCCTGAAGTTTGGGTCAGGATATGTGTCTGGATGGGGAAGAGTCTTCCACAAGGGCAGG
TCTGCACTGGTGCTGCAGTATCTGAGAGTGCCTCTGGTGGATAGGGCCACTTGTCTGCTGTCTACCAAGTTCACCATC
TACAACAATATGTTCTGTGCTGGATTTCATGAGGGAGGGAGAGACTCCTGTCAGGGAGATTCTGGAGGCCCACATGTG
ACAGAGGTGGAAGGCACCAGCTTCCTGACAGGCATCATTTCCTGGGGGGAGGAATGTGCAATGAAGGGGAAATATGGA
ATCTACACCAAAGTGAGCAGGTATGTGAACTGGATCAAGGAAAAGACCAAACTGACATGA

In vitro expression of the liver-optimized fIX sequence (SEQ ID NO: 10) and human-optimized fIX sequence (SEQ ID NO: 127) was assessed in HepG2 cells transiently transfected with corresponding fVIII expression plasmids (FIG. 7). The tissue specific optimization lead to increased fIX activity in the HepG2 (hepatic) cells expressing the liver-optimized fIX compared to human-optimized fIX. These results show that liver optimization specifically benefits expression in hepatocyte derived cells.

Example 2

Promoter Development

This example described the iterative development of optimized promoter sequences for expression of protein in liver tissue and cells.

The promoters were synthesized *de novo* and cloned into an expression plasmid driving the expression of clotting fVIII. FVIII activity was measured 48 hours after transfection by one-stage clot assay. As a comparator, the hybrid liver promoter (HLP) was used. HLP represents one of the shortest yet most powerful liver-directed promoters described to date. The HLP promoter and its use are described in McIntosh et al., "Therapeutic levels of FVIII following a single peripheral vein administration of rAAV vector encoding a novel human fVIII variant," Blood, 25;121(17):3335-44, 2013. The HLP sequence is provided as SEQ ID NO: 128:

TGTTTGCTGCTTGCAATGTTTGCCCATTTTAGGGTGGACACAGGACGCTGTGGTTTCTGAGCCAGGGGGCGACTCAGA
TCCCAGCCAGTGGACTTAGCCCCTGTTTGCTCCTCCGATAACTGGGGTGACCTTGGTTAATATTCACCAGCAGCCTCC
CCCGTTGCCCCTCTGGATCCACTGCTTAAATACGGACGAGGACAGGGCCCTGTCTCCTCAGCTTCAGGCACCACCACT
GACCTGGGACAGTGAATC

Initial Promoter Design (1st Generation Promoters)

To begin construction of synthetic liver-directed promoters, two minimal liver directed promoters were selected that were designed platforms for further modification. These two promoters are designated as "ABP-SynO" (SEQ ID NO: 131) and "ABP-HP1-God". These promoters are novel fusions of previously described regulatory control elements. "ABP" is clustered region of transcription factor binding sites, "HP1" is a specific transcription factor binding site, "God" is an enhancer-like region that functions in direct proximity to the transcription start site, and "SynO" is a minimal promoter that contains the HP1 transcription factor binding site and a TATA box. For all constructs, where not provided within the native context, a TATA sequence (TATAAA) was added or completed immediately 3' to the promoter region.

Initial promoter designed were based on the "ABP" element, which is described, for example, in Rouet et al., "A potent enhancer made of clustered liver-specific elements in the transcription control sequences of human alpha 1-microglobulin/bikunin gene," J Biol Chem., 267(29):20765-73, 1992. ABP comprises the nucleotide sequence set forth as

ABP element (SEQ ID NO: 113):

GTTAATTTTTAAAAAGCAGTCAAAAGTCCAAGTGGCCCTTGCGAGCATTTACTCTCTCTGTTTGCTCTGGTTAATAAT
CTCAGGAGCACAAACA

As illustrated in FIG. 8, ABP comprises the following TF binding sites: HNF-1-1 (nucleotides 16-23 of SEQ ID NO: 4), HNF-4 (nucleotides 26-36 of SEQ ID NO: 4), HNF-3a (nucleotides 39-45 of SEQ ID NO: 4), HNF1-2 (nucleotides 48-62 of SEQ ID NO: 4), and HNF-3-2 TF (nucleotides 65-71 of SEQ ID NO: 4).

Several of the disclosed promoters include an HP1 TF binding site (GTTAATAATTTTC, nucleotides 75-87 of SEQ ID NO: 4). The HP1 element is described, for example, in Schorpp et al., "Hepatocyte-specific promoter element HP1 of the Xenopus albumin gene interacts with transcriptional factors of mammalian hepatocytes," J Mol Biol., 202(2):307-20, 1988. The HP1 TF binding site is included in the SynO element (included in several of the disclosed promoters), which also includes a TATA box. The sequence of the SynO element is provided as

SynO element

GAGGTTAATAATTTTCCAGATCTCTCTGAGCAATAGTATAAAA (SEQ ID NO: 114)

The SynO element is described, for example, in Ryffel et al., "Liver cell specific gene transcription *in vitro*: the promoter elements HP1 and TATA box are necessary and sufficient to generate a liver-specific promoter." Nucleic Acids Res., 17(3): 939–953, 1989.

Several of the disclosed promoters include a "God" which comprises the sequence set forth as:

God element

AGTCATATGTTTGCTCACTGAAGGTTACTAGTTAACAGGCATCCCTTAAACAGGA (SEQ ID NO: 115)

The God element is described, for example, in Godbout et al., "Multiple regulatory elements in the intergenic region between the alpha-fetoprotein and albumin genes," Mol Cell Biol., 6(2):477-87, 1986.

The ABP, SynO, and God elements were combined to form two novel promoters, "ABP-SynO" and "ABP-HP1-God" as follows (see FIG. 8):

ABP-SynO promoter (SEQ ID NO: 131)

GTTAATTTTTAAAAAGCAGTCAAAAGTCCAAGTGGCCCTTGCGAGCATTTACTCTCTCTGTTTGCTCTGGTTAATAAT
CTCAGGAGCACAAACAGAGGTTAATAATTTTCCAGATCTCTCTGAGCAATAGTATAAAA

ABP-Hp1-God promoter (SEQ ID NO: 6)

GTTAATTTTTAAAAAGCAGTCAAAAGTCCAAGTGGCCCTTGCGAGCATTTACTCTCTCTGTTTGCTCTGGTTAATAAT
CTCAGGAGCACAAACAGAGGTTAATAATTTTCAGTCATATGTTTGCTCACTGAAGGTTACTAGTTAACAGGCATCCCT
TAAACAGGATATAAAA

In vitro expression of FVIII was assayed in HepG2 cells transiently transfected with FVIII expression plasmids driven by the ABP-SynO or ABP-Hp1-God promoter (FIG. 9A). Additionally, *In vivo* fVIII activity in mice was assayed by hydrodynamically delivered naked plasmid expressing FVIII driven by the ABP-SynO or ABP-Hp1-God promoter (FIG. 9B). From the results, it is shown that both the ABP-Syno and ABP-Hp1-God promoters drive fVIII expression *in vitro* and *in vivo*. However, these initial designs do not drive fVIII expression as strongly as the HLP promoter *in vitro*, and that the expression *in vivo* declines rapidly relative to HLP.

Initial Optimization (2nd Generation Promoters)

In order to increase the transcriptional strength of the ABP-SynO and ABP-Hp1-God promoters, multiple strategies for optimization were pursued. This includes altering the transcription factor binding sites to reflect the consensus binding sequence, removing intervening space between transcription factor binding sites, adding additional transcription factor binding sites, adding a transcription start site motif, and including the SV40 intron.

An ABP variant was generated that contains consensus TF binding sites, as follows:

ABP-exact (consensus transcription factor binding sites) (SEQ ID NO: 116)

GTTAATCATTAACTTAAAAAGCAGTCAAAAGTCCAAAGGTCAAAGGTCAGAGCATTTACTCTCTCCAATGTTGACTCT
CGTTAATGATTAAGGAGCAATTGTTGACTT

As illustrated in FIG. 8, ABP-exact comprises the following consensus TF binding sites: consensus HNF-1-1, consensus HNF-4, consensus HNF-3a, consensus HNF1-2, and consensus HNF-3-2. A condensed version of Apb-exact was generated that includes the same consensus TF binding sites, but a shorter overall sequence, termed Short-ABP-exact, the sequence of which is set forth as:

Short-ABP-exact (SEQ ID NO: 117)

GTTAATCATTAACTTAGGTCAAAGGTCAGACAATGTTGACTCTCGTTAATGATTAACCGGAATTGTTGACTT

The following features were further included in certain of the disclosed promoters:

A transcription start site (TSS), which contains a 23 contains a GC rich spacer and was placed immediately after a TATA box in the promoter for optimal spacing with the transcription start motif immediately after the spacer (see FIG. 10). The TSS sequence assayed includes the sequence set forth as: GCCAGCAGCAGCCTGACCACATCTCATCCTC (nucleotides 116-146 of SEQ ID NO: 4)

A HNF1a transcription factor binding site. HNF1a is a liver-directed transcription factor: GTTAATCATTAA (nucleotides 1-12 of SEQ ID NO: 4)

A Sp1 transcription factor binding site. Sp1 is a liver-directed transcription factor: TGGGCGGAGT (nucleotides 1-10 of SEQ ID NO: 121)

A SV40 intron sequence set forth as:

CTCTAAGGTAAATATAAAATTTTTAAGTGTATAATGTGTTAAACTACTGATTCTAATTGTTTGTGTATTTTAGATTCC
AACCTATGGAACTGA (nucleotides 163-225 of SEQ ID NO: 112)

These elements were combined to form several novel promoters, as follows (see FIG. 11):

ABP-exact-SynO (SEQ ID NO: 118)

GTTAATCATTAACTTAAAAAGCAGTCAAAAGTCCAAAGGTCAAAGGTCAGAGCATTTACTCTCTCCAATGTTGACTCT
CGTTAATGATTAAGGAGCAATTGTTGACTTGAGGTTAATAATTTTCCAGATCTCTCTGAGCAATAGTATAAAA

ShortABP-exact-SynO (SEQ ID NO: 119)

GTTAATCATTAACTTAGGTCAAAGGTCAGACAATGTTGACTCTCGTTAATGATTAACCGGAATTGTTGACTTGAGGTT
AATAATTTTCCAGATCTCTCTGAGCAATAGTATAAAA

ABP-HP1-God-TSS (SEQ ID NO: 7)

GTTAATTTTTAAAAAGCAGTCAAAAGTCCAAGTGGCCCTTGCGAGCATTTACTCTCTCTGTTTGCTCTGGTTAATAAT
CTCAGGAGCACAAACAGAGGTTAATAATTTTCAGTCATATGTTTGCTCACTGAAGGTTACTAGTTAACAGGCATCCCT
TAAACAGGATATAAAAGGCCAGCAGCAGCCTGACCACATCTCATCCTC

HNF1a-ABP-SynO (SEQ ID NO: 120)

GTTAATCATTAAGTCGTTAATTTTTAAAAAGCAGTCAAAAGTCCAAGTGGCCCTTGCGAGCATTTACTCTCTCTGTTT
GCTCTGGTTAATAATCTCAGGAGCACAAACAGAGGTTAATAATTTTCCAGATCTCTCTGAGCAATAGTATAAAA

Sp1-ABP-SynO (SEQ ID NO: 121)

TGGGCGGAGTGTCGTTAATTTTTAAAAAGCAGTCAAAAGTCCAAGTGGCCCTTGCGAGCATTTACTCTCTCTGTTTGC
TCTGGTTAATAATCTCAGGAGCACAAACAGAGGTTAATAATTTTCCAGATCTCTCTGAGCAATAGTATAAAA

HNF1-ShortABPExact-SynO-TSS-Int (SEQ ID NO: 112)

GTTAATCATTAAGTCGTTAATCATTAACTTAGGTCAAAGGTCAGACAATGTTGACTCTCGTTAATGATTAACCGGAAT
TGTTGACTTGAGGTTAATAATTTTCCAGATCTCTCTGAGCAATAGTATAAAAGGCCAGCAGCAGCCTGACCACATCTC
ATCCTCCTCTAAGGTAAATATAAAATTTTTAAGTGTATAATGTGTTAAACTACTGATTCTAATTGTTTGTGTATTTTA
GATTCCAACCTATGGAACTGA

The promoters were synthesized *de novo* and cloned into an expression plasmid driving the expression of clotting fVIII. FVIII activity was measured 48 hours after transfection by one-stage clot assay. As a comparator, the hybrid liver promoter (HLP) is used in this and other experiments.

In vitro expression of FVIII was assayed in HepG2 cells transiently transfected with FVIII expression plasmids driven by the respective promoter (FIG. 12A). Additionally, *In vivo* fVIII activity in mice was assayed by hydrodynamically delivered naked plasmid expressing FVIII driven by the respective promoter (FIG. 12B). From these data, it is shown that the addition of the synthetic, novel, transcription start site motif substantially improves expression. Further, the addition of transcription factor binding sites outside of their native genomic context had unexpected and unpredicted impacts on expression. When added directly proximal to the ABP enhancer element, the HNF1 and Sp1 transcription factor binding sites completely abrogated expression. When the HNF1 transcription factor binding site was added directly proximal to the shortABPExact enhancer, expression was robust. This finding illustrates that the complex spatiotemporal interactions of transcription factors, in either their native or non-native genomic context, is difficult to model or predict. In addition, altering the transcription factor binding sites to the consensus binding sequences abrogated gene expression. However, the addition of the HNF1 transcription factor binding site, transcription start site, and SV40 intron was sufficient to rescue expression from HNF1 supplemented shortABPExact-SynO promoter design. As illustrated in FIG. 12B, the ABP-Hp1-God-TSS and the HNF1-shortABPExact-SynO-TSS-Int both maintained higher expression than the HLP promoter over the course of the experiment, showing that the addition of the transcription start site, HNF1 transcription factor binding site, adjustment of the ABP sequence to the consensus sequence and removal of intervening DNA between transcription factor binding sites, and/or the addition of the SV40 intron could improve the durability and strength of expression *in vivo*.

Further Optimization (3rd Generation Promoters)

While the ABP-Hp1-God-TSS promoter design tested far exceeded the strength of the HLP promoter, its size (204 bae pairs) remained incompatible with some complete packaging of AAV-vectors, such as those containing full-length containing fVIII transgenes. Further reduction in the size of the promoter was targeted by selection of the most promising elements tested and described above, as well as a novel element, shortABP, which is the ABP enhancer where the native genomic transcription factor binding site sequences are retained, but the intervening sequences between them have been truncated.

shortABP (nucleotides 16-71 of SEQ ID NO: 4):

GTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACA

As illustrated in FIG. 13, shortABP contains the following TF binding sites: HNF-1-1 (nucleotides 16-23 of SEQ ID NO: 4), HNF-4 (nucleotides 26-36 of SEQ ID NO: 4), HNF-3a (nucleotides 39-45 of SEQ ID NO: 4), HNF1-2 (nucleotides 48-62 of SEQ ID NO: 4), and HNF-3-2 TF (nucleotides 65-71 of SEQ ID NO: 4).

As illustrated in FIG. 13, the HNF1 TF binding site, the SynO element (containing a HP1 TF binding site and a TATA box), and a transcription start site were linked to the shortABP to form the HNF1-shortABP-SynO-TSS (designated the Hepatic Combinatorial Bundle, or HCB), as follows:

HNF1-shortABP-SynO-TSS (also called Hepatic Combinatorial Bundle, or HCB) (SEQ ID NO: 4)

GTTAATCATTAAGTCGTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACAGAGGTTA
ATAATTTTCCAGATCTCTCTGAGCAATAGTATAAAAGGCCAGCAGCAGCCTGACCACATCTCATCCTC

As illustrated in FIG. 13, the HNF1 TF binding site, the God element, a TATA box, and a transcription start site were linked to the shortABP to form the shortABP-HP1-God-TSS, as follows:

shortABP-HP1-God-TSS (SEQ ID NO: 5)

GTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACATACATTTTCAGTCATATGTTTG
CTCACTGAAGGTTACTAGTTAACAGGCATCCCTTAAACAGGATATAAAAGGCCAGCAGCAGCCTGACCACATCTCATC
CTC

The promoters were synthesized *de novo* and cloned into an expression plasmid driving the expression of clotting fVIII. FVIII activity was measured 48 hours after transfection by one-stage clot assay. *In vitro* expression of FVIII was assayed in HepG2 cells transiently transfected with FVIII expression plasmids driven by the respective promoter is shown in FIG. 14A. Additionally, *In vivo* fVIII activity in mice was assayed by hydrodynamically delivered naked plasmid expressing FVIII driven by the respective promoter (FIG. 14). From these data, it is shown that the HNF1-shortABP-SynO-TSS promoter (HCB) promotes increased expression of fVIII compared to that of HLP both *in vivo* and *in vitro*, while maintaining durable expression 4-14 fold greater than that of HLP *in vivo*, *despite the HNF1-shortABP-SynO-TSS being 42% smaller than HLP.*

Supplemental Optimization (4th Generation Promoters)

A powerful, liver-directed enhancer, designated the hepatocyte specific computational regulatory module (HSCRM8 or "HS") was recently constructed by combining the sequences from several species into a computationally constructed novel enhancer *(see, e.g.,* Nair et al., "Computationally designed liver-specific transcriptional modules and hyperactive fIX improve hepatic gene therapy," Blood, 23(20): 3195–3199, 2014). The sequence of the HS enhancer and corresponding transcription factor binding sites are provided as follows:

HS (HSCRM8 non-human enhancer sequence) (SEQ ID NO: 101)

GGGGAGGCTGCTGGTGAATATTAACCAAGGTCACCCCAGTTATCGGAGGAGCAAACAGGGACTAAGTCCAC

The HS response element includes the following TF binding sites:

MYOD	GGCTGCTGGTGAATATT, nucleotides 5-22 of SEQ ID NO: 101
CEBP	GCTGCTGGTGAA, nucleotides 7-18 of SEQ ID NO: 101
Nhf1	GCTGGTGAATATTAACCA, nucleotides 10-27 of SEQ ID NO: 101
Lef1/TCF1	TTAACCAAGGT, nucleotides 21-31 of SEQ ID NO: 101
CEBP	CGGAGGAGCAAA, nucleotides 44-55 of SEQ ID NO: 101
Forkhead	GGAGCAAACAGGG, nucleotides 48-60 of SEQ ID NO: 101
Lef1/TCF1	AGGGACTAAG, nucleotides 57-66 of SEQ ID NO: 101

The human genome was examined to determine human sequences corresponding to those of the HS element and the HS element was modified to contain only human sequences to generate a fully human enhancer sequence termed "HSh." The sequence of the HSh enhancer and corresponding transcription factor binding sites are provided as follows (see also, FIG. 15):

HSh (human genomic sequence) (SEQ ID NO: 111)

GGGGAGGCTGCTGGTGAATATTAACCAAGGTCACCCCAGTTATCGGAGGAGCAAACAGGG**G**CTAAGTCCAC

The HSh response element includes the following TF binding sites:

MYOD	GGCTGCTGGTGAATATT, nucleotides 5-22 of SEQ ID NO: 101
CEBP	GCTGCTGGTGAA, nucleotides 7-18 of SEQ ID NO: 101
Nhf1	GCTGGTGAATATTAACCA, nucleotides 10-27 of SEQ ID NO: 101
Lef1/TCF1	TTAACCAAGGT, nucleotides 21-31 of SEQ ID NO: 101
CEBP	CGGAGGAGCAAA, nucleotides 44-55 of SEQ ID NO: 101
Forkhead	GGAGCAAACAGGG, nucleotides 48-60 of SEQ ID NO: 101
Lef1/TCF1	AGGG**G**CTAAG, nucleotides 57-66 of SEQ ID NO: 111

Portions of the HSh enhancer were also utilized, as follows:

5'HSh (5' portion of HSCRM8h) (nucleotides 6-32 of SEQ ID NO: 111)

GGCTGCTGGTGAATATTAACCAAGGTC

The 5'HSh response element includes the following TF binding sites:

MYOD	GGCTGCTGGTGAATATT, nucleotides 5-22 of SEQ ID NO: 101
CEBP	GCTGCTGGTGAA, nucleotides 7-18 of SEQ ID NO: 101
Nhf1	GCTGGTGAATATTAACCA, nucleotides 10-27 of SEQ ID NO: 101
Lef1/TCF1	TTAACCAAGGT, nucleotides 21-31 of SEQ ID NO: 101

3'HSh (3' portion of HSCRM8h) (nucleotides 44-68 of SEQ ID NO: 111)

CGGAGGAGCAAACAGGG**G**CTAAGTC

The 3'HSh response element includes the following TF binding sites:

CEBP	CGGAGGAGCAAA, nucleotides 44-55 of SEQ ID NO: 101
Forkhead	GGAGCAAACAGGG, nucleotides 48-60 of SEQ ID NO: 101
Lef1/TCF1	AGGG**G**CTAAG nucleotides 57-66 of SEQ ID NO: 111

sHS (short HS, the intervening space between the 5' and 3' clusters of transcription factor binding sites has been removed) (nucleotides 1-54 of SEQ ID NO: 106)

GGCTGCTGGTGAATATTAACCAAGGTCATCGGAGGAGCAAACAGGGACTAAGTC

The sHS response element includes the following TF binding sites:

MYOD	GGCTGCTGGTGAATATT, nucleotides 5-22 of SEQ ID NO: 101
CEBP	GCTGCTGGTGAA, nucleotides 7-18 of SEQ ID NO: 101
Nhf1	GCTGGTGAATATTAACCA, nucleotides 10-27 of SEQ ID NO: 101
Lef1/TCF1	TTAACCAAGGT, nucleotides 21-31 of SEQ ID NO: 101
CEBP	CGGAGGAGCAAA, nucleotides 44-55 of SEQ ID NO: 101
Forkhead	GGAGCAAACAGGG, nucleotides 48-60 of SEQ ID NO: 101
Lef1/TCF1	AGGGACTAAG, nucleotides 57-66 of SEQ ID NO: 111

Additionally, a modified form of the short ABP promoter, termed "supershortABP" was constructed by further deleting nucleotides and rearranging TF binding sites. The sequence of the supershort ABP response element and corresponding transcription factor binding sites are provided as follows:

Super short ABP (further shortened ABP-based element) (SEQ ID NO: 122)

CCCTTGCTGGTTAATAATCTCAGTTAATTTGTTTGCACAAACA

The supershort ABP response element include the following TF binding sites:

HNF4	CCCTTGC, nucleotides 1-7 of SEQ ID NO: 122
HNF1b	TGGTTAATAATCTCA, nucleotides 8-22 of SEQ ID NO: 122
HNF1	GTTAATT, nucleotides 23-29 of SEQ ID NO: 122
HNF3	TGTTTGC, nucleotides 30-36 of SEQ ID NO: 122
HNF3b	ACAAACA, nucleotides 37-43 of SEQ ID NO: 122

As illustrated in FIG. 16, the above elements were combined to form several novel promoters, as follows (see FIG. 11):

Agro (SEQ ID NO: 107)

GGCTGCTGGTGAATATTAACCAAGGTCCCCTTGCTGGTTAATAATCTCAGTTAATTTGTTTGCACAAACACGGAGGAG
CAAACAGGGGAGGTTAATAATTTTCTATAAAAGGCCAGCAGCAGCCTGACCACATCTCATCCTC

The Agro sequence includes 5'HS (nucleotides 1-27 of SEQ ID NO: 107), Super short ABP (nucleotides 28-70 of SEQ ID NO: 107), 3'HSh (nucleotides 71-87 of SEQ ID NO: 107), SynO (nucleotides 88-110 of SEQ ID NO: 107), and TSS (nucleotides 111-142 of SEQ ID NO: 107)

HSh-HCB (SEQ ID NO: 102)

GGGGAGGCTGCTGGTGAATATTAACCAAGGTCACCCCAGTTATCGGAGGAGCAAACAGGGGCTAAGTCCACTAGGTTA
ATCATTAAGTCGTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACAGAGGTTAATAA
TTTTCCAGATCTCTCTGAGCAATAGTATAAAAGGCCAGCAGCAGCCTGACCACATCTCATCCTC

5'HSh-HCB (SEQ ID NO: 104)

GGCTGCTGGTGAATATTAACCAAGGTCGTTAATCATTAAGTCGTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGT
TAATAATCTCAGGACAAACAGAGGTTAATAATTTTCCAGATCTCTCTGAGCAATAGTATAAAAGGCCAGCAGCAGCCT
GACCACATCTCATCCTC

3'HSh-HCB (SEQ ID NO: 103)

CGGAGGAGCAAACAGGGGCTAAGTCGTTAATCATTAAGTCGTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTA
ATAATCTCAGGACAAACAGAGGTTAATAATTTTCCAGATCTCTCTGAGCAATAGTATAAAAGGCCAGCAGCAGCCTGA
CCACATCTCATCCTC

HSh-SynO-TSS (SEQ ID NO: 105)

GGGGAGGCTGCTGGTGAATATTAACCAAGGTCACCCCAGTTATCGGAGGAGCAAACAGGGGCTAAGTCCACGAGGTTA
ATAATTTTCCAGATCTCTCTGAGCAATAGTATAAAAGGCCAGCAGCAGCCTGACCACATCTCATCCTC

HS-SynO-TSS (SEQ ID NO: 108)

GGGGAGGCTGCTGGTGAATATTAACCAAGGTCACCCCAGTTATCGGAGGAGCAAACAGGGACTAAGTCCACGAGGTTA
ATAATTTTCCAGATCTCTCTGAGCAATAGTATAAAAGGCCAGCAGCAGCCTGACCACATCTCATCCTC

sHS-SynO-TSS (SEQ ID NO: 106)

GGCTGCTGGTGAATATTAACCAAGGTCATCGGAGGAGCAAACAGGGACTAAGTCGAGGTTAATAATTTTCCAGATCTC
TCTGAGCAATAGTATAAAAGGCCAGCAGCAGCCTGACCACATCTCATCCTC

The promoters were synthesized *de novo* and cloned into an expression plasmid driving the expression of clotting fVIII. FVIII activity was measured 48 hours after transfection by one-stage clot assay. *In vitro* expression of FVIII was assayed in HepG2 cells transiently transfected with FVIII expression plasmids driven by the respective promoter is shown in FIG. 17. These results demonstrate that the *in vivo* strength of the HCB promoter can be augmented by the addition of a supplemental enhancer sequences. The greatest benefit was seen when the complete, human, HSh enhancer was added to the 5' end of the HCB promoter. Supplementing only the 5' or 3' portions of the HSh attenuated the improved expression slightly compared to the complete HSh module. Additionally, when the shortABP enhancer was removed from the HCB design and instead replaced with either HSh or HS enhancers, fVIII expression declined slightly from the levels seen in the HSh-HCB promoter, which contains the shortABP-enhancer. However, when the sHS-SynO plasmid was administered hydrodynamically to mice, no expression was observed, showing that the shortABP enhancer module may be a more suitable enhancer for durable *in vivo* expression.

Example 3

Recombinant AAV vector for fVIII Expression

This example illustrates exemplary recombinant AAV vectors encoding a fVIII variant that comprise a genome sized for optimal AAV vector-based protein expression (that is, a genome of 5 kb or fewer bp).

FIG. 4 depicts AAV-based vectors for expression of fVIII variants that lack the B-domain. However, the constructs shown in FIG. 4 are above the 5.0 kb limit for optimal protein expression from an AAV vector. Removing nonessential viral genomic DNA and cloning remnants, and substituting shorter promoter sequences (such as the HCB promoter (SEQ ID NO: 4, 146 bp), enabled the development of a high expression fVIII-AAV genome of approximately 4.9kb in length.

An exemplary AAV vector with such a genome is depicted in FIG. 18. This figure illustrates an AAV vector including 5'and 3' ITRs, the HCB (SEQ ID NO: 4) promoter, a nucleic acid molecule encoding a variant fVIII protein that lacks the B-domain (such as any one of the ET3 or HSQ CpG-depleted and liver codon optimized sequences provided herein), and a synthetic poly A sequence.

An exemplary sequence of an AAV cassette as shown in FIG. 18 is provided as SEQ ID NO: 129, which has the following structure:

(5'AAV2 ITR) – *RE* – (HCB Promoter) – Kozak – (HSQ coding region) – *RE* – (poly adenylation signal) – *RE* – (3'AAV2 ITR)

The elements of the AAV cassette of SEQ ID NO: 129 are as follows:

Element	Start (bp)	End (bp)
5' AAV2 ITR	1	141
AgeI (restriction site)	142	147
HCB promoter	148	293
Destroyed XhoI site	294	299
Kozak consensus sequence	300	304
Liver optimized HSQ with CpGs	305	4678
NotI (restriction site)	4679	4686
Rabbit beta globin polyA signal	4687	4735
MunI (restriction site)	4736	4741
3' AAV2 ITR	4742	4882

The restriction sites can optionally be removed from the cassette to provide a shortened recombinant AAV genome. Additionally, the transgene can be substituted as needed. Removing the restriction sites elements would generate a vector of 4885 base pairs (ET3) or 4855 (HSQ).

SEQ ID NO: 129:

CCTGCAGGCAGCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGCC
CGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGCCAACTCCATCACTAGGGGTTCCTACCGGTGTTAATCAT
TAAGTCGTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACAGAGGTTAATAATTTTC
CAGATCTCTCTGAGCAATAGTATAAAAGGCCAGCAGCAGCCTGACCACATCTCATCCTCGTCGAGCCACCATGCAGAT
CGAACTGTCTACCTGTTTCTTTCTGTGCCTGCTGCGGTTTTGTTTTTCCGCTACCAGAAGATACTACCTGGGAGCCGT
CGAACTGAGCTGGGATTACATGCAGTCTGACCTGGGAGAGCTGCCCGTGGACGCTAGATTCCCACCTAGAGTCCCTAA
GTCCTTCCCCTTCAACACCAGCGTGGTCTACAAGAAAACCCTGTTCGTGGAGTTTACCGACCACCTGTTCAACATCGC
TAAGCCTAGACCACCATGGATGGGACTGCTGGGACCAACCATCCAGGCCGAGGTGTACGACACCGTGGTCATCACCCT
GAAAAACATGGCTTCTCACCCCGTGTCCCTGCATGCTGTGGGCGTCTCCTACTGGAAGGCCAGCGAAGGGGCTGAGTA
TGACGATCAGACCAGCCAGCGGGAAAAAGAGGACGATAAGGTGTTCCCTGGCGGGTCCCATACCTACGTGTGGCAGGT
CCTGAAGGAGAATGGACCAATGGCTTCCGACCCTCTGTGCCTGACCTACTCTTATCTGTCCCACGTGGACCTGGTCAA
GGATCTGAACAGCGGCCTGATCGGGGCTCTGCTGGTGTGTCGCGAAGGGTCCCTGGCCAAGGAGAAAACCCAGACCCT
GCATAAGTTCATCCTGCTGTTCGCCGTGTTTGACGAAGGAAAAAGCTGGCACTCTGAGACCAAGAACTCTCTGATGCA
GGACAGGGATGCCGCTTCCGCCAGAGCTTGGCCCAAGATGCACACCGTGAACGGCTACGTCAATAGGAGCCTGCCTGG

ACTGATCGGCTGCCACAGAAAGTCCGTGTATTGGCATGTCATCGGAATGGGCACCACCCCTGAAGTGCACAGCATCTT
CCTGGAGGGGCATACCTTTCTGGTCCGCAACCACCGGCAGGCTAGCCTGGAGATCTCTCCAATCACCTTCCTGACCGC
CCAGACCCTGCTGATGGACCTGGGACAGTTCCTGCTGTTTTGCCACATCTCCAGCCACCAGCATGATGGCATGGAGGC
TTACGTGAAAGTCGACTCCTGTCCCGAGGAACCTCAGCTGAGGATGAAGAACAATGAGGAAGCCGAAGACTATGACGA
TGACCTGACCGACAGCGAGATGGATGTGGTCCGCTTCGATGACGATAACTCTCCCTCCTTTATCCAGATCCGGTCCGT
GGCCAAGAAACACCCTAAGACCTGGGTCCATTACATCGCCGCTGAGGAAGAGGACTGGGATTATGCTCCACTGGTGCT
GGCCCCCGACGATAGATCCTACAAAAGCCAGTATCTGAACAATGGACCCCAGAGGATCGGCAGAAAGTACAAGAAAGT
GAGGTTCATGGCTTATACCGATGAGACCTTTAAGACCAGAGAAGCCATCCAGCACGAGTCCGGGATCCTGGGACCTCT
GCTGTACGGCGAAGTGGGGGACACCCTGCTGATCATCTTCAAGAACCAGGCCAGCAGGCCTTACAATATCTATCCACA
TGGCATCACCGATGTGAGACCTCTGTACTCCCGCCGGCTGCCAAAGGGCGTGAAACACCTGAAGGACTTCCCAATCCT
GCCCGGGGAAATCTTTAAGTATAAATGGACCGTCACCGTCGAGGATGGGCCCACCAAGAGCGACCCTAGGTGCCTGAC
CAGATACTATTCTTCCTTCGTGAATATGGAGAGAGACCTGGCTTCCGGACTGATCGGACCCCTGCTGATCTGTTACAA
AGAGAGCGTGGATCAGCGCGGCAACCAGATCATGTCTGACAAGCGGAATGTGATCCTGTTCAGCGTCTTTGACGAAAA
CCGCTCTTGGTACCTGACCGAGAACATCCAGCGGTTCCTGCCTAATCCAGCTGGAGTGCAGCTGGAAGATCCCGAGTT
CCAGGCCTCTAACATCATGCATTCCATCAATGGCTACGTGTTCGACTCCCTGCAGCTGAGCGTGTGCCTGCACGAGGT
CGCTTACTGGTATATCCTGAGCATCGGAGCCCAGACCGATTTCCTGTCTGTGTTCTTTTCCGGCTACACCTTTAAGCA
TAAAATGGTGTATGAGGACACCCTGACCCTGTTCCCATTTTCCGGCGAAACCGTGTTCATGAGCATGGAGAATCCCGG
GCTGTGGATCCTGGGATGCCACAACTCCGATTTCAGGAATAGAGGGATGACCGCCCTGCTGAAAGTGAGCTCTTGTGA
CAAGAACACCGGAGACTACTATGAAGATAGCTACGAGGACATCTCTGCTTATCTGCTGTCCAAAAACAATGCCATCGA
GCCCAGGAGCTTCTCTCAGAACCCTCCAGTGCTGAAGCGCCACCAGCGGGAGATCACCAGAACCACCCTGCAGAGCGA
TCAGGAAGAGATCGACTACGACGATACCATCTCCGTGGAAATGAAGAAAGAGGACTTCGATATCTATGACGAAGATGA
GAACCAGTCTCCCAGGTCCTTCCAGAAGAAAACCAGACATTACTTTATCGCCGCTGTGGAGCGGCTGTGGGACTATGG
CATGTCCAGCTCTCCTCACGTGCTGAGAAATAGAGCTCAGTCCGGAAGCGTCCCACAGTTCAAGAAAGTGGTCTTCCA
GGAGTTTACCGACGGAAGCTTTACCCAGCCACTGTACCGCGGCGAACTGAACGAGCACCTGGGGCTGCTGGGACCCTA
TATCCGGGCTGAAGTGGAGGATAACATCATGGTCACCTTCAGGAATCAGGCCAGCAGACCCTACTCTTTTTATTCCAG
CCTGATCTCCTACGAAGAGGACCAGAGACAGGGAGCTGAACCAAGAAAAAACTTCGTGAAGCCTAATGAGACCAAAAC
CTACTTTTGGAAGGTGCAGCACCATATGGCCCCTACCAAAGACGAGTTCGATTGCAAGGCCTGGGCTTATTTTAGCGA
CGTGGATCTGGAGAAGGACGTCCACTCCGGCCTGATCGGGCCACTGCTGGTGTGTCATACCAACACCCTGAATCCAGC
TCACGGAAGGCAGGTGACCGTCCAGGAATTCGCCCTGTTCTTTACCATCTTTGATGAGACCAAGAGCTGGTACTTCAC
CGAAAACATGGAGAGGAATTGCAGAGCCCCATGTAACATCCAGATGGAAGACCCCACCTTCAAGGAGAACTACAGATT
TCATGCTATCAATGGGTATATCATGGATACCCTGCCAGGACTGGTCATGGCTCAGGACCAGAGGATCAGATGGTACCT
GCTGAGCATGGGGTCTAACGAGAATATCCACTCCATCCATTTCAGCGGACACGTGTTTACCGTCCGCAAGAAAGAAGA
GTACAAGATGGCCCTGTACAACCTGTATCCCGGCGTGTTCGAAACCGTCGAGATGCTGCCTTCCAAGGCTGGGATCTG
GCGGGTGGAATGCCTGATCGGGGAGCACCTGCATGCCGGAATGTCTACCCTGTTCCTGGTGTACTCCAATAAGTGTCA
GACCCCCCTGGGGATGGCTAGCGGACATATCCGCGACTTCCAGATCACCGCTTCCGGACAGTACGGACAGTGGGCTCC
TAAGCTGGCTAGACTGCACTATTCTGGCTCCATCAACGCTTGGTCTACCAAAGAGCCTTTCTCCTGGATCAAGGTGGA
CCTGCTGGCTCCAATGATCATCCATGGCATCAAAACCCAGGGGGCCAGGCAGAAGTTCTCTTCCCTGTACATCAGCCA
GTTTATCATCATGTATTCTCTGGATGGGAAGAAATGGCAGACCTACAGAGGCAATTCCACCGGGACCCTGATGGTGTT
CTTTGGCAACGTCGACAGCTCTGGGATCAAGCACAACATCTTCAATCCCCCTATCATCGCCCGCTACATCCGGCTGCA
CCCAACCCATTATTCCATCCGCAGCACCCTGCGGATGGAGCTGATGGGGTGCGATCTGAACAGCTGTTCTATGCCCCT
GGGAATGGAGTCTAAGGCCATCTCCGACGCTCAGATCACCGCCTCCAGCTACTTCACCAATATGTTTGCTACCTGGTC
CCCAAGCAAGGCTAGACTGCATCTGCAGGGAAGAAGCAACGCTTGGAGACCACAGGTGAACAATCCCAAGGAGTGGCT
GCAGGTCGACTTCCAGAAAACCATGAAGGTGACCGGAGTCACCACCCAGGGCGTGAAAAGCCTGCTGACCTCTATGTA
CGTCAAGGAGTTCCTGATCTCTTCCAGCCAGGACGGGCACCAGTGGACCCTGTTCTTTCAGAACGGAAAGGTGAAAGT
CTTCCAGGGCAATCAGGATTCCTTTACCCCTGTGGTCAACAGCCTGGACCCACCCCTGCTGACCAGGTACCTGAGAAT
CCACCCACAGTCCTGGGTGCATCAGATCGCTCTGAGGATGGAAGTCCTGGGCTGCGAGGCCCAGGACCTGTATTGAGC
GGCCGCAATAAAATATCTTTATTTTCATTACATCTGTGTGTTGGTTTTTTGTGTGCAATTGAGGAACCCCTAGTGATG
GAGTTGGCCACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCCGGGCGACCAAAGGTCGCCCGACGCCCGGGCTTT
GCCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGCAGCTGCCTGCAGG

Another exemplary AAV vector including a fVIII transgene driven by the HCB promoter is provided as SEQ ID NO: 130, which provides a prototypical design of an AAV cassette encoding a therapeutic transgene under control of the HCB promoter. Each element is separated by one or two restriction enzyme (RE) sites, which allow for easy substitution of these elements. In SEQ ID NO: 130, the order of elements is as follows:

(5'AAV2 ITR) – *RE* – (HCB Promoter) – *RE* – (MVM Intron) – *RE* – Kozak – (ET3 coding region) – *RE* – (poly adenylation signal) – *RE* – (3'AAV2 ITR)

AAV2-HCB-ET3-LCO-NCG-SpA

Element	Start (bp)	End (bp)
5' AAV2 ITR	1	141
AgeI (restriction site)	142	147
HCB promoter	148	293
SalI + PacI (restriction sites)	294	307
MVM intron	308	399
XhoI (restriction site)	400	405
Kozak consensus sequence	406	410
Liver optimized ET3 no CpGs	411	4814
NotI (restriction site)	4815	4822
Rabbit beta globin polyA signal	4823	4871
MunI (restriction site)	4872	4877
3' AAV2 ITR	4878	5018

The intron and restriction sites can optionally be removed from the cassette to provide a shortened recombinant AAV genome. Additionally, the transgene can be substituted as needed. Removing the intron and restriction sites elements would generate a vector of 4885 base pairs (ET3) or 4855 (HSQ).

AAV2-HCB-ET3-LCO-NCG-SpA virus particles were generated and used to transduce mice. fVIII activity in serum was assayed at various time point post-transduction (see FIG. 19). The *in vivo* gene therapy assay was performed substantially as described in Brown et al. ("Bioengineered Factor FVIII Enables Long-Term Correction of Murine Hemophilia A Following Liver-Directed Adeno-Associated Viral Vector Delivery," *Molecular Therapy – Methods and Clinical Development.* 1:14036, 2014).

SEQ ID NO: 130:

CCTGCAGGCAGCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGCC
CGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGCCAACTCCATCACTAGGGGTTCCTACCGGTGTTAATCAT
TAAGTCGTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACAGAGGTTAATAATTTTC
CAGATCTCTCTGAGCAATAGTATAAAAGGCCAGCAGCAGCCTGACCACATCTCATCCTCGTCGACTTAATTAAAAGAG
GTAAGGGTTTAAGGGATGGTTGGTTGGTGGGGTATTAATGTTTAATTACCTGGAGCACCTGCCTGAAATCACTTTTTT
TCAGGTTGGCTCGAGCCACCATGCAGCTGGAACTGTCTACCTGTGTGTTTCTGTGTCTGCTGCCTCTGGGGTTTTCTG
CTATCAGGAGATACTATCTGGGAGCTGTGGAGCTGTCCTGGGACTACAGGCAGTCTGAGCTGCTGAGAGAACTGCATG
TGGATACCAGATTCCCAGCTACAGCTCCAGGAGCTCTGCCTCTGGGCCCATCTGTGCTGTACAAGAAAACAGTCTTTG
TGGAGTTTACAGACCAGCTGTTCTCTGTGGCCAGGCCAAGACCACCTTGGATGGGACTGCTGGGACCAACCATCCAGG
CTGAGGTGTATGATACAGTGGTGGTGACCCTGAAAAACATGGCCTCCCATCCTGTGAGCCTGCATGCTGTGGGGGTGT
CCTTCTGGAAGTCCTCTGAGGGAGCTGAGTATGAAGACCATACCTCCCAGAGGGAGAAAGAAGATGATAAGGTGCTGC
CTGGCAAAAGCCAGACCTATGTCTGGCAGGTGCTGAAGGAGAATGGACCAACTGCTTCTGACCCACCATGCCTGACCT
ACTCTTATCTGTCCCATGTGGATCTGGTGAAGGACCTGAATTCTGGACTGATTGGAGCTCTGCTGGTGTGTAGAGAGG
GAAGCCTGACCAGAGAAAGAACCCAGAACCTGCATGAGTTTGTCCTGCTGTTTGCTGTGTTTGATGAAGGGAAGAGCT
GGCACTCTGCCAGGAATGACTCCTGGACCAGAGCTATGGATCCAGCTCCTGCTAGAGCTCAGCCTGCTATGCACACAG
TCAATGGCTATGTGAATAGGTCTCTGCCAGGACTGATTGGCTGCCATAAGAAATCTGTCTATTGGCATGTGATTGGAA
TGGGCACCAGCCCTGAGGTGCATTCTATCTTCCTGGAAGGCCACACCTTTCTGGTCAGGCACCATAGACAGGCCTCTC
TGGAGATCTCCCCTCTGACCTTCCTGACAGCTCAGACCTTTCTGATGGACCTGGGGCAGTTCCTGCTGTTTTGCCATA

TCTCTTCCCACCATCATGGAGGAATGGAGGCTCATGTCAGGGTGGAATCCTGTGCTGAGGAACCACAGCTGAGAAGAA
AGGCTGATGAGGAAGAGGACTATGATGATAACCTGTATGACTCTGATATGGATGTGGTGAGGCTGGATGGGGATGATG
TCAGCCCTTTCATCCAGATCAGGTCTGTGGCCAAGAAACATCCAAAGACCTGGGTCCACTACATTGCTGCTGAAGAGG
AAGATTGGGACTATGCCCCCCTGGTGCTGGCTCCTGATGATAGATCCTACAAAAGCCAGTATCTGAACAATGGGCCCC
AGAGGATTGGAAGGAAGTACAAGAAAGTGAGGTTCATGGCCTATACAGATGAGACCTTTAAGACCAGAGAGGCTATCC
AGCATGAATCTGGGATCCTGGGACCTCTGCTGTATGGAGAAGTGGGGGATACCCTGCTGATCATCTTCAAGAACCAGG
CCTCCAGGCCATACAATATCTATCCCCATGGCATCACAGATGTGAGACCACTGTACAGCAGGAGACTGCCCAAGGGGG
TCAAACACCTGAAGGATTTCCCCATCCTGCCTGGAGAGATCTTTAAGTATAAATGGACAGTCACAGTGGAAGATGGGC
CTACCAAGTCTGATCCAAGGTGCCTGACCAGATACTATAGCTCTTTTGTGAACATGGAGAGAGACCTGGCTTCTGGAC
TGATTGGACCCCTGCTGATCTGTTACAAAGAGTCTGTGGACCAGAGGGGCAACCAGATCATGTCTGATAAGAGAAATG
TCATCCTGTTCTCTGTGTTTGATGAGAACAGGAGCTGGTACCTGACAGAGAACATCCAGAGGTTCCTGCCAAATCCAG
CTGGAGTGCAGCTGGAGGACCCAGAATTTCAGGCTTCCAACATCATGCATAGCATCAATGGCTATGTGTTTGATAGCC
TGCAGCTGTCTGTCTGCCTGCATGAGGTGGCCTACTGGTATATCCTGTCCATTGGAGCTCAGACAGACTTCCTGTCTG
TGTTCTTTAGTGGGTACACCTTTAAGCATAAAATGGTGTATGAGGATACCCTGACCCTGTTCCCCTTTTCTGGGGAGA
CAGTGTTCATGTCCATGGAAAACCCTGGCCTGTGGATCCTGGGGTGCCACAACTCTGACTTCAGGAATAGAGGAATGA
CAGCCCTGCTGAAAGTGTCCAGCTGTGATAAGAATACAGGGGATTACTATGAGGACTCTTATGAAGATATCTCTGCTT
ATCTGCTGAGCAAGAACAATGCCATTGAGCCCAGGTCTTTTGCTCAGAACTCCAGACCTCCATCTGCTTCTGCTCCTA
AGCCACCTGTGCTGAGAAGACATCAGAGGGACATCTCCCTGCCTACCTTCCAGCCAGAGGAAGATAAAATGGACTATG
ATGATATCTTCAGCACAGAGACCAAGGGGGAAGATTTTGACATCTATGGAGAGGATGAAAACCAGGATCCAAGATCCT
TCCAGAAGAGAACCAGACACTACTTTATTGCTGCTGTGGAGCAGCTGTGGGACTATGGGATGTCTGAAAGCCCAAGGG
CCCTGAGGAACAGAGCTCAGAATGGAGAGGTGCCCAGATTCAAGAAAGTGGTGTTCAGAGAGTTTGCTGATGGCAGCT
TTACCCAGCCATCTTACAGGGGGGAGCTGAACAAGCATCTGGGGCTGCTGGGACCCTATATCAGAGCTGAGGTGGAAG
ATAACATCATGGTGACCTTCAAGAATCAGGCTTCTAGGCCCTACTCCTTTTATTCTTCCCTGATCTCCTACCCTGATG
ATCAGGAGCAGGGAGCTGAACCTAGGCACAACTTTGTGCAGCCAAATGAGACCAGAACCTACTTTTGGAAGGTGCAGC
ATCACATGGCTCCCACAGAGGATGAATTTGACTGCAAAGCTTGGGCCTATTTTTCTGATGTGGACCTGGAGAAGGATG
TGCATTCTGGCCTGATTGGGCCTCTGCTGATCTGTAGGGCCAACACCCTGAATGCTGCTCATGGAAGACAGGTCACAG
TGCAGGAGTTTGCTCTGTTCTTTACCATCTTTGATGAAACCAAGAGCTGGTACTTCACAGAGAATGTGGAAAGGAATT
GCAGAGCCCCCTGTCATCTGCAGATGGAGGACCCTACCCTGAAGGAAAACTACAGGTTCCATGCCATCAATGGATATG
TCATGGATACCCTGCCTGGCCTGGTCATGGCTCAGAACCAGAGGATCAGATGGTACCTGCTGTCTATGGGATCCAATG
AGAATATCCATAGCATCCACTTCTCTGGCCATGTCTTTTCTGTGAGGAAGAAAGAGGAATACAAAATGGCTGTGTACA
ATCTGTATCCTGGGGTCTTTGAGACAGTGGAAATGCTGCCAAGCAAAGTGGGAATCTGGAGAATTGAGTGCCTGATTG
GGGAACACCTGCAGGCTGGGATGAGCACCACCTTCCTGGTGTACTCTAAGAAATGTCAGACCCCACTGGGGATGGCCT
CTGGACATATCAGGGACTTCCAGATCACAGCTTCTGGACAGTATGGACAGTGGGCTCCAAAGCTGGCTAGACTGCACT
ATTCTGGCTCCATCAATGCCTGGTCTACCAAAGAGCCATTCTCCTGGATCAAGGTGGACCTGCTGGCCCCCATGATCA
TCCATGGAATCAAAACCCAGGGAGCTAGGCAGAAGTTCAGCTCTCTGTACATCTCCCAGTTTATCATCATGTATAGCC
TGGATGGGAAGAAATGGCAGACCTACAGAGGCAATTCCACTGGGACCCTGATGGTCTTCTTTGGAAATGTGGATTCCT
CTGGCATCAAGCACAACATCTTCAATCCACCCATCATTGCCAGGTACATCAGGCTGCATCCTACCCACTATAGCATCA
GGTCTACCCTGAGAATGGAGCTGATGGGATGTGACCTGAACAGCTGTTCTATGCCACTGGGCATGGAGTCCAAGGCTA
TCTCTGATGCCCAGATCACAGCTTCTTCCTACTTCACCAATATGTTTGCTACCTGGTCCCCAAGCAAGGCTAGACTGC
ACCTGCAGGGAAGATCCAATGCTTGGAGACCCCAGGTGAACAATCCTAAGGAGTGGCTGCAGGTGGACTTCCAGAAAA
CCATGAAGGTCACAGGGGTGACCACCCAGGGAGTGAAATCTCTGCTGACCTCCATGTATGTCAAGGAGTTCCTGATCA
GCTCTTCCCAGGATGGCCACCAGTGGACCCTGTTCTTTCAGAATGGCAAGGTCAAAGTGTTCCAGGGGAATCAGGACT
CTTTTACCCCAGTGGTGAACTCCCTGGATCCTCCACTGCTGACCAGGTACCTGAGAATCCATCCTCAGAGCTGGGTGC
ACCAGATTGCTCTGAGAATGGAGGTCCTGGGATGTGAAGCTCAGGACCTGTATTGAGCGGCCGCAATAAAATATCTTT
ATTTTCATTACATCTGTGTGTTGGTTTTTTGTGTGCAATTGAGGAACCCCTAGTGATGGAGTTGGCCACTCCCTCTCT
GCGCGCTCGCTCGCTCACTGAGGCCGGGCGACCAAAGGTCGCCCGACGCCCGGGCTTTGCCCGGGCGGCCTCAGTGAG
CGAGCGAGCGCGCAGCTGCCTGCAGG

Example 4

Treatment of human hemophilia A using AAV-based gene therapy

This example describes an exemplary method for the clinical use of AAV vectors encoding fVIII for the treatment of hemophilia A.

A patient diagnosed with hemophilia A is selected for treatment. The patient is administered a therapeutically effective amount of a recombinant AAV encoding the ET3 or HSQ fVIII variant, such as AAV-ET3 or AAV-HSQ under control of a HCB promoter as disclosed herein. The recombinant AAV can be administered intravenously. An appropriate therapeutic dose can be selected by a medical

practitioner. In some cases, the therapeutically effective dose is in the range of 1×10^{11} to 1×10^{14} viral particles (vp)/kg, such as about 1×10^{12} vp/kg. In most instances, the patient is administered a single dose. The health of the subject can be monitored over time to determine the effectiveness of the treatment.

It will be apparent that the precise details of the methods or compositions described may be varied or modified without departing from the spirit of the described embodiments. We claim all such modifications and variations that fall within the scope and spirit of the claims below.

We Claim:

1. A recombinant nucleic acid molecule, comprising:

a liver-specific promoter comprising a first response element of no more than 160 nucleotides in length that comprises:

a HNF1a transcription factor (TF) binding site;

a HNF1-1 TF binding site;

a HNF4 TF binding site;

a HNF3a TF binding site;

a HNF1-2 TF binding site;

a HNF3-2 TF binding site;

a HP1 TF binding site

a TATA box; and

a Transcription Start Site.

2. The recombinant nucleic acid molecule of claim 1, wherein:

the HNF1a TF binding site comprises or consists of nucleotides 1-12 of SEQ ID NO: 4;

the HNF1-1 TF binding site comprises or consists of nucleotides 16-23 of SEQ ID NO: 4;

the HNF4 TF binding site comprises or consists of nucleotides 26-36 of SEQ ID NO: 4;

the HNF3a TF binding site comprises or consists of nucleotides 39-45 of SEQ ID NO: 4;

the HNF1-2 TF binding site comprises or consists of nucleotides 48-62 of SEQ ID NO: 4;

the HNF3-2 TF binding site comprises or consists of nucleotides 65-71 of SEQ ID NO: 4;

the HP1 TF binding site comprises or consists of nucleotides 75-87 of SEQ ID NO: 4;

the TATA box comprises or consists of nucleotides 108-114 of SEQ ID NO: 4; and

the Transcription Start Site (TSS) comprises or consists of nucleotides 116-146 of SEQ ID NO: 4.

3. The recombinant nucleic acid molecule of claim 1 or claim 2, wherein the first response element comprises, from 5' to 3', the HNF1a TF binding site, the HNF1-1 TF binding site, the HNF4 TF binding site, the HNF3a TF binding site, the HNF1-2 TF binding site, the HNF3-2 TF binding site, the HP1 TF binding site, the TATA box, and the Transcription Start Site (TSS).

4. The recombinant nucleic acid molecule of any one of the prior claims, wherein the first response element is no more than 150 nucleotides in length.

5. The recombinant nucleic acid molecule of any one of the prior claims, wherein the first response element is 146 nucleotides in length.

6. The recombinant nucleic acid molecule of any one of the prior claims, wherein the first response element comprises or consists of a nucleotide sequence at least 90% identical to SEQ ID NO: 4.

7. The recombinant nucleic acid molecule of claim 6, wherein the first response element comprises or consists of the nucleotide sequence set forth as SEQ ID NO: 4.

8. The recombinant nucleic acid molecule of any of the prior claims, wherein the promoter consists of the first response element.

9. The recombinant nucleic acid molecule of any one of claims 1-8, further comprising a second response element upstream of the first response element, the second response element comprising one of:

a HSh response element comprising or consisting of a nucleotide sequence at least 90% identical to SEQ ID NO: 111;

a 5'HS response element comprising or consisting of a nucleotide sequence at least 90% identical to nucleotides 6-32 of SEQ ID NO: 111; or

a 3'HS response element comprising or consisting of a nucleotide sequence at least 90% identical to nucleotides 44-68 of SEQ ID NO: 111.

10. The recombinant nucleic acid molecule of claim 9, wherein the

the HSh response element comprises or consists of the nucleotide sequence set forth as SEQ ID NO: 111;

the 5'HS response element comprises or consists of nucleotides 6-32 of SEQ ID NO: 111; or

the 3'HS response element comprises or consists of nucleotides 44-68 of SEQ ID NO: 111.

11. The recombinant nucleic acid molecule of claim 10, wherein the

the HSh response element consists of the nucleotide sequence set forth as SEQ ID NO: 111;

the 5'HS response element consists of nucleotides 6-32 of SEQ ID NO: 111; or

the 3'HS response element consists of nucleotides 44-68 of SEQ ID NO: 111.

12. The recombinant nucleic acid molecule of any of claims 9-11, wherein the recombinant promoter comprises or consists of a nucleotide sequence at least 90% identical to one of SEQ ID NO: 102 (HSh-HCB), SEQ ID NO: 104 (5'HSh-HCB), or SEQ ID NO: 103 (3'HSh-HCB).

13. The recombinant nucleic acid molecule of claims 12, wherein the recombinant promoter comprises or consists of the nucleotide sequence set forth as SEQ ID NO: 102 (HSh-HCB), SEQ ID NO: 104 (5'HSh-HCB), or SEQ ID NO: 103 (3'HSh-HCB).

14. A recombinant nucleic acid molecule, comprising or consisting of a nucleotide sequence at least 90% identical to any one of SEQ ID NO: 5 (shortABP-HP1-God-TSS), SEQ ID NO: 7 (ABP-HP1-God-TSS), SEQ ID NO: 105 (HSh-SynO-TSS), SEQ ID NO: 106 (sHS-SynO-TSS), SEQ ID NO:

107 (Agro), SEQ ID NO: 108 (HS-SynO-TSS), or SEQ ID NO: 112 (HNF1-ShortABPExact-SynO-TSS-Int).

15. The recombinant nucleic acid molecule of claim 14, comprising or consisting of the nucleotide sequence set forth as any one of SEQ ID NO: 5 (shortABP-HP1-God-TSS), SEQ ID NO: 7 (ABP-HP1-God-TSS), SEQ ID NO: 105 (HSh-SynO-TSS), SEQ ID NO: 106 (sHS-SynO-TSS), SEQ ID NO: 107 (Agro), SEQ ID NO: 108 (HS-SynO-TSS), or SEQ ID NO: 112 (HNF1-ShortABPExact-SynO-TSS-Int).

16. A vector comprising the recombinant nucleic acid molecule of any one of the prior claims.

17. The vector of claim 16, wherein the vector is a viral vector.

18. The vector of claim 17, wherein the viral vector is an AAV vector.

19. The vector of claim 16, wherein the viral vector is a gamma-retroviral vector, a lentiviral vector, or an adenoviral vector.

20. The vector of any one of claims 16-19, comprising a nucleic acid molecule encoding a clotting factor that is operably linked to the promoter.

21. The vector of claim 20, wherein the clotting factor is fVIII, or an ET3 or B domain deleted human (HSQ) fVIII variant.

22. The vector of claim 21, wherein the fVIII comprises the nucleic acid sequence set forth as SEQ ID NO: 2, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 125, or SEQ ID NO: 126, or a nucleic acid sequence at least 90% identical to SEQ ID NO: 2, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 125, or SEQ ID NO: 126.

23. The vector of claim 20, wherein the clotting factor is fIX

24. The vector of claim 23, wherein the fIX comprises the nucleic acid sequence set forth as SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 124, or SEQ ID NO: 127, or a nucleic acid sequence at least 90% identical SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 124, or SEQ ID NO: 127.

25. A composition comprising the vector of any one of claims 16-24 in a pharmaceutically acceptable carrier.

26. An isolated nucleic acid molecule, comprising:

a nucleic acid sequence encoding fVIII and comprising the nucleotide sequence set forth as one of SEQ ID NO: 2, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 125, or SEQ ID NO: 126, or a nucleotide sequence at least 90% identical to one of SEQ ID NO: 2, SEQ ID NO: 11, SEQ ID NO: 12 SEQ ID NO: 125, or SEQ ID NO: 126; or

a nucleic acid sequence encoding fIX and comprising the nucleotide sequence set forth as one of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 124, or SEQ ID NO: 127, or a nucleotide sequence at least 90% identical to one of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 124, or SEQ ID NO: 127.

27. A vector comprising the recombinant nucleic acid molecule of claim 26 operably linked to a promoter.

28. The vector of claim 27, wherein the vector is a viral vector.

29. The vector of claim 28, wherein the viral vector is an AAV vector.

30. The vector of claim 28, wherein the viral vector is a gamma-retroviral vector, a lentiviral vector, or an adenoviral vector.

31. A composition comprising the vector of any one of claims 27-30 in a pharmaceutically acceptable carrier.

32. A method of inducing blood clotting in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of the vector of any one of claims 20-24 or 27-30, or the composition of claim 25 or claim 31.

33. A method of treating a subject with a clotting disorder, comprising selecting a subject with the clotting disorder and administering to the subject a therapeutically effective amount of the vector of any one of claims 20-24 or 27-30, or the composition of claim 25 or claim 31.

34. The method of claim 33, wherein the clotting disorder is:

hemophilia A and the subject is administered a vector comprising a nucleic acid molecule encoding a protein with fVIII activity; or

hemophilia B and the subject is administered a vector comprising a nucleic acid molecule encoding a protein with fIX activity.

35. The method of any one of claims 32-34, wherein the vector is administered intravenously.

36. The method of any one of claims 32-34, wherein the vector is an AAV vector.

37. The method of any one of claims 32-36, wherein the vector is administered in combination with an immunosuppressive agent.

38. The method of claim 37, wherein the immunosuppressive agent is ciclosporin, tacrolimus, sirolimus, cyclophosphamide, methotrexate, azathioprine, mercaptopurine, fluorouracil, mycophenolic acid, dactinomycin, fingolimod, T-cell receptor antibody or binding protein, muromonab-CD3, IL-2 receptor antibody or binding protein, basiliximab, daclizumab, recombinant IFN-beta, TNF-alpha antibody or binding protein, infliximab, etanercept, or adalimumab.

39. Use of the vector of any one of claims 20-24 or 27-30, or the composition of claim 25 or claim 31, to inducing blood clotting in a subject in need thereof or treating a subject with a clotting disorder.

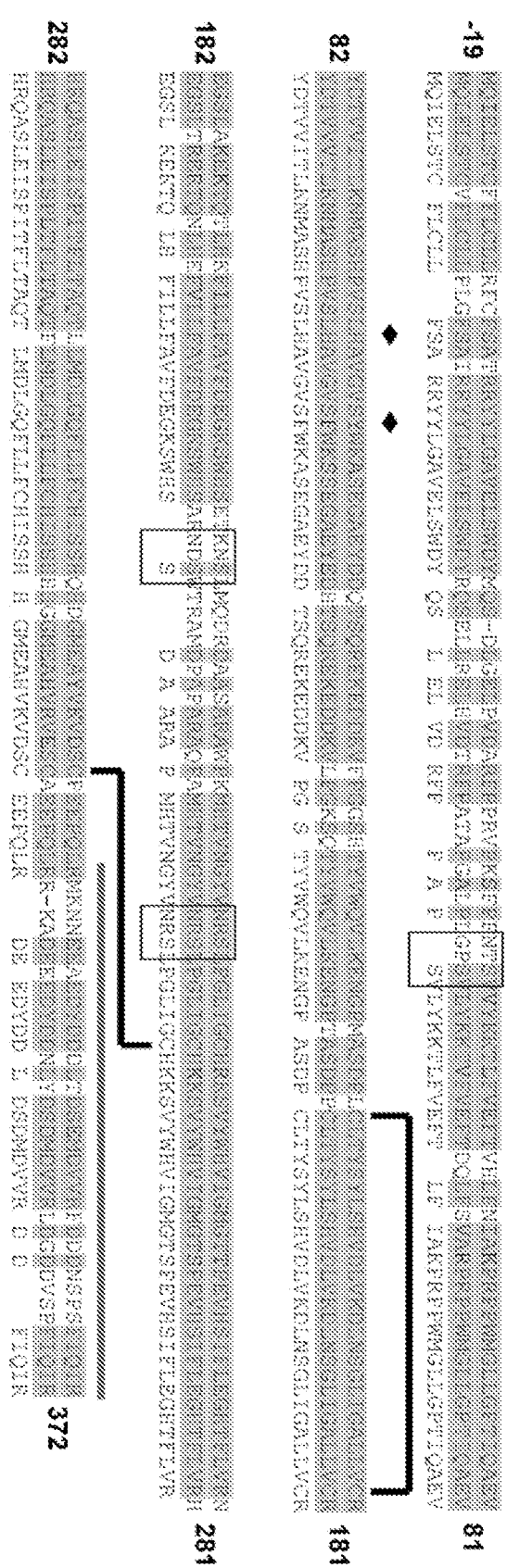


FIG. 1A

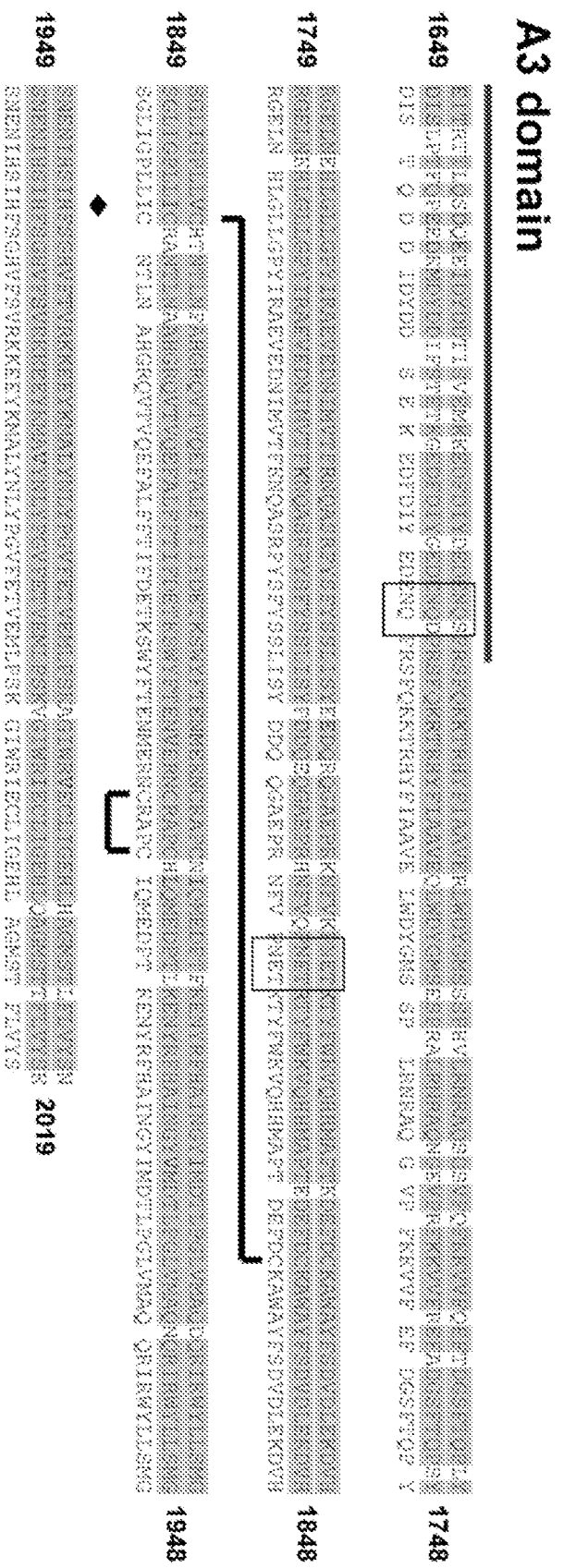


FIG. 1B

Liver-specific human codon optimization table

AA	Codon	Total Frequency	Liver Frequency	Liver:Total
Ala	GCC	0.4	0.44	1.10
Ala	GCA	0.23	0.19	0.83
Ala	GCT	0.26	0.27	1.04
Ala	GCG	0.11	0.1	0.91
Arg	CGC	0.19	0.24	1.26
Arg	AGA	0.2	0.18	0.90
Arg	CGA	0.11	0.1	0.91
Arg	CGT	0.08	0.07	0.88
Arg	AGG	0.2	0.2	1.00
Arg	CGG	0.21	0.21	1.00
Asn	AAC	0.54	0.59	1.09
Asn	AAT	0.46	0.41	0.89
Asp	GAC	0.54	0.61	1.13*
Asp	GAT	0.46	0.39	0.85
Cys	TGC	0.55	0.6	1.09
Cys	TGT	0.45	0.4	0.89
Gln	CAA	0.25	0.25	1.00
Gln	CAG	0.75	0.75	1.00
Glu	GAG	0.58	0.65	1.12*
Glu	GAA	0.42	0.35	0.83
Gly	GGC	0.34	0.37	1.09
Gly	GGT	0.16	0.12	0.78
Gly	GGG	0.25	0.28	1.12
Gly	GGA	0.25	0.23	0.92
His	CAC	0.59	0.59	1.00
His	CAT	0.41	0.41	1.00
Ile	ATC	0.48	0.61	1.27*
Ile	ATT	0.36	0.28	0.78
Ile	ATA	0.16	0.11	0.69
Leu	CTG	0.41	0.47	1.15
Leu	CTC	0.2	0.23	1.15*
Leu	CTT	0.13	0.1	0.77
Leu	TTA	0.07	0.04	0.57
Leu	TTG	0.13	0.11	0.85
Leu	CTA	0.07	0.06	0.86
Lys	AAG	0.58	0.65	1.12*
Lys	AAA	0.42	0.35	0.83
Met	ATG	1	1	1.00
Phe	TTC	0.55	0.65	1.18*
Phe	TTT	0.45	0.35	0.78*
Pro	CCC	0.33	0.38	1.15
Pro	CCA	0.27	0.24	0.89
Pro	CCG	0.11	0.1	0.91
Pro	CCT	0.28	0.28	1.00
Ser	TCC	0.22	0.28	1.27
Ser	AGT	0.15	0.12	0.80
Ser	TCA	0.15	0.13	0.87
Ser	AGC	0.24	0.25	1.04
Ser	TCT	0.18	0.17	0.94
Ser	TCG	0.06	0.06	1.00
Thr	ACC	0.36	0.47	1.31*
Thr	ACA	0.28	0.21	0.75
Thr	ACT	0.24	0.19	0.79
Thr	ACG	0.12	0.12	1.00
Trp	TGG	1	1	1.00
Tyr	TAC	0.57	0.63	1.11
Tyr	TAT	0.43	0.37	0.86
Val	GTG	0.47	0.52	1.11
Val	GTT	0.18	0.13	0.72
Val	GTA	0.11	0.08	0.73
Val	GTC	0.24	0.26	1.09

FIG. 2A

Standard human codon optimization table

Codon	Frequency	Count	Codon	Frequency	Count	Codon	Frequency	Count	Codon	Frequency	Count
UUU	0.464134	714298	UCU	0.187586	618711	UAU	0.443338	495699	UGU	0.456157	430311
UUC	0.535866	824692	UCC	0.21796	718892	UAC	0.556662	622407	UGC	0.543843	513028
UUA	0.076568	311881	UCA	0.150517	496448	UAA	0	0	UGA	1	10000
UUG	0.129058	525688	UCG	0.054398	179419	UAG	0	0	UGG	1	535595
CUU	0.131716	536515	CCU	0.286731	713233	CAU	0.418515	441711	CGU	0.080108	184609
CUC	0.195577	796638	CCC	0.32347	804620	CAC	0.581485	613713	CGC	0.183777	423516
CUA	0.07138	290751	CCA	0.276603	688038	CAA	0.265017	501911	CGA	0.108812	250760
CUG	0.395702	1611801	CCG	0.113196	281570	CAG	0.734983	1391973	CGG	0.201554	464485
AUU	0.361072	650473	ACU	0.246769	533609	AAU	0.470367	689701	AGU	0.149602	493429
AUC	0.469866	846466	ACC	0.355232	768147	AAC	0.529633	776603	AGC	0.239938	791383
AUA	0.169062	304565	ACA	0.284188	614523	AAA	0.434049	993621	AGA	0.214658	494682
AUG	1	896005	ACG	0.113812	246105	AAG	0.565951	1295568	AGG	0.211091	486463
GUU	0.18177	448607	GCU	0.265922	750096	GAU	0.464542	885429	GGU	0.163087	437126
GUC	0.238306	588138	GCC	0.399781	1127679	GAC	0.535458	1020595	GGC	0.337109	903565
GUA	0.116577	287712	GCA	0.228121	643471	GAA	0.422453	1177632	GGA	0.249922	669873
GUG	0.463346	1143534	GCG	0.106176	299495	GAG	0.577547	1609975	GGG	0.249882	669768

FIG. 2B

Monocyte/Myeloid Codon optimization tables

Codon	Frequency	Count	Codon	Frequency	Count	Codon	Frequency	Count	Codon	Frequency	Count
UUU	0.39	95	UCU	0.15	72	UAU	0.4	70	UGU	0.33	52
UUC	0.61	150	UCC	0.22	103	UAC	0.6	107	UGC	0.67	106
UUA	0.05	27	UCA	0.17	77	UAA	0	0	UGA	1	100
UUG	0.12	67	UCG	0.04	17	UAG	0	0	UGG	1	92
CUU	0.1	56	CCU	0.22	75	CAU	0.39	54	CGU	0.1	27
CUC	0.24	139	CCC	0.38	127	CAC	0.61	83	CGC	0.14	37
CUA	0.07	40	CCA	0.31	104	CAA	0.28	71	CGA	0.09	25
CUG	0.43	251	CCG	0.09	30	CAG	0.72	180	CGG	0.18	48
AUU	0.26	66	ACU	0.21	78	AAU	0.43	121	AGU	0.14	66
AUC	0.6	155	ACC	0.42	153	AAC	0.57	161	AGC	0.28	131
AUA	0.14	37	ACA	0.27	98	AAA	0.37	130	AGA	0.22	58
AUG	1	137	ACG	0.1	36	AAG	0.63	225	AGG	0.27	71
GUU	0.16	56	GCU	0.32	114	GAU	0.46	137	GGU	0.13	56
GUC	0.29	103	GCC	0.35	124	GAC	0.54	161	GGC	0.37	153
GUA	0.09	32	GCA	0.25	88	GAA	0.44	180	GGA	0.24	98
GUG	0.47	169	GCG	0.07	25	GAG	0.56	230	GGG	0.26	109

FIG. 2C

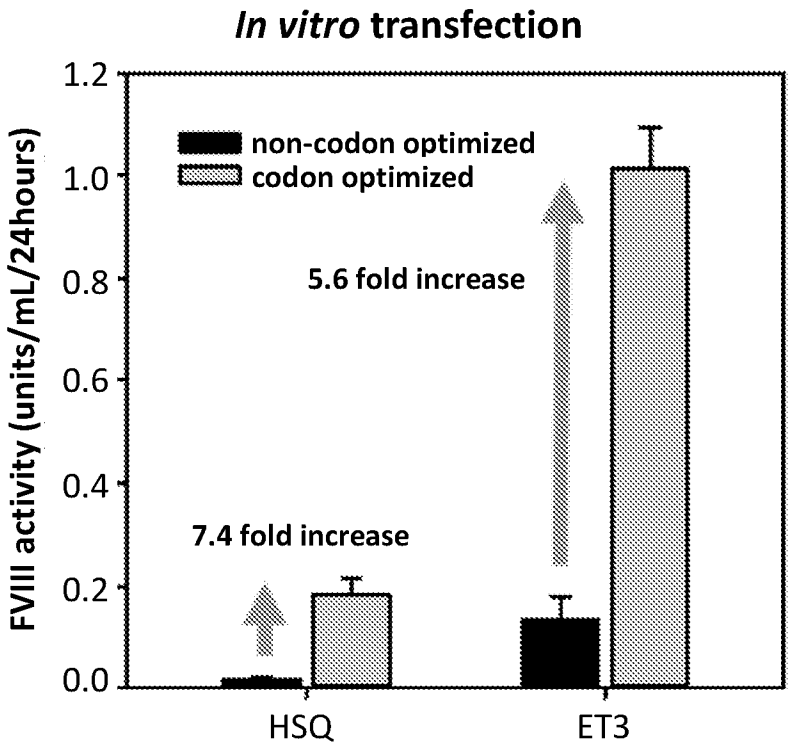

In vitro transfection
FVIII activity (units/mL/24hours)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
non-codon optimized
codon optimized
5.6 fold increase
7.4 fold increase
HSQ
ET3

FIG. 3A

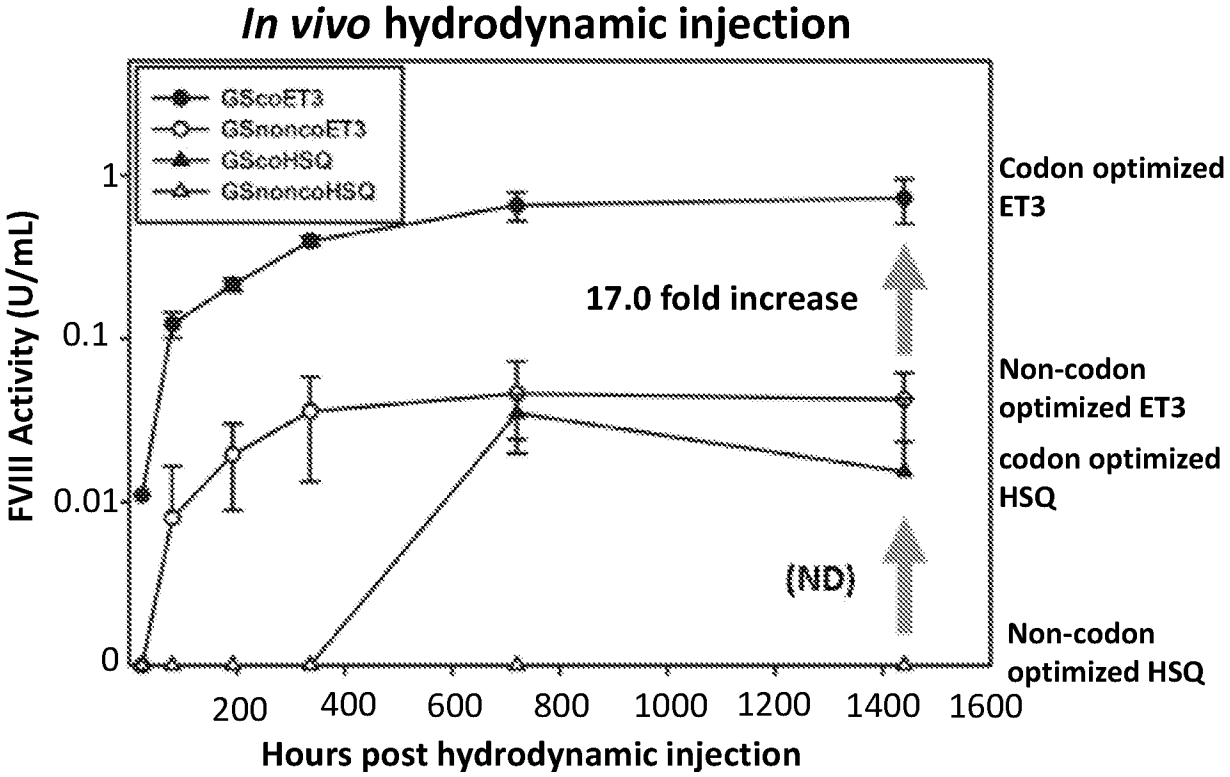

In vivo hydrodynamic injection
GScoET3
GSnoncoET3
GScoHSQ
GSnoncoHSQ
FVIII Activity (U/mL)
1
0.1
0.01
0
17.0 fold increase
(ND)
Codon optimized ET3
Non-codon optimized ET3
codon optimized HSQ
Non-codon optimized HSQ
200
400
600
800
1000
1200
1400
1600
Hours post hydrodynamic injection

FIG. 3B

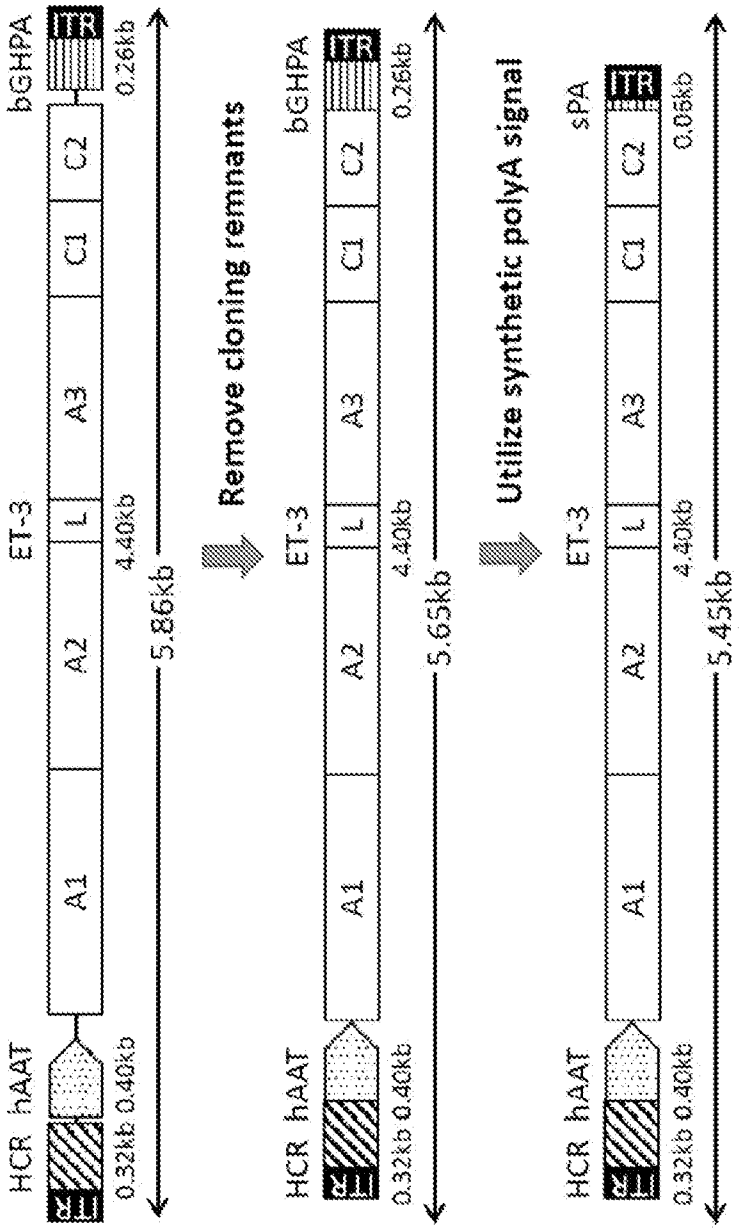

HCR
hAAT
ITR
0.32kb
0.40kb
A1
A2
L
ET-3
4.40kb
A3
C1
C2
bGHPA
ITR
0.26kb
5.86kb
Remove cloning remnants
HCR
hAAT
ITR
0.32kb
0.40kb
A1
A2
L
ET-3
4.40kb
A3
C1
C2
bGHPA
ITR
0.26kb
5.65kb
Utilize synthetic polyA signal
HCR
hAAT
ITR
0.32kb
0.40kb
A1
A2
L
ET-3
4.40kb
A3
C1
C2
sPA
ITR
0.05kb
5.45kb

FIG. 4

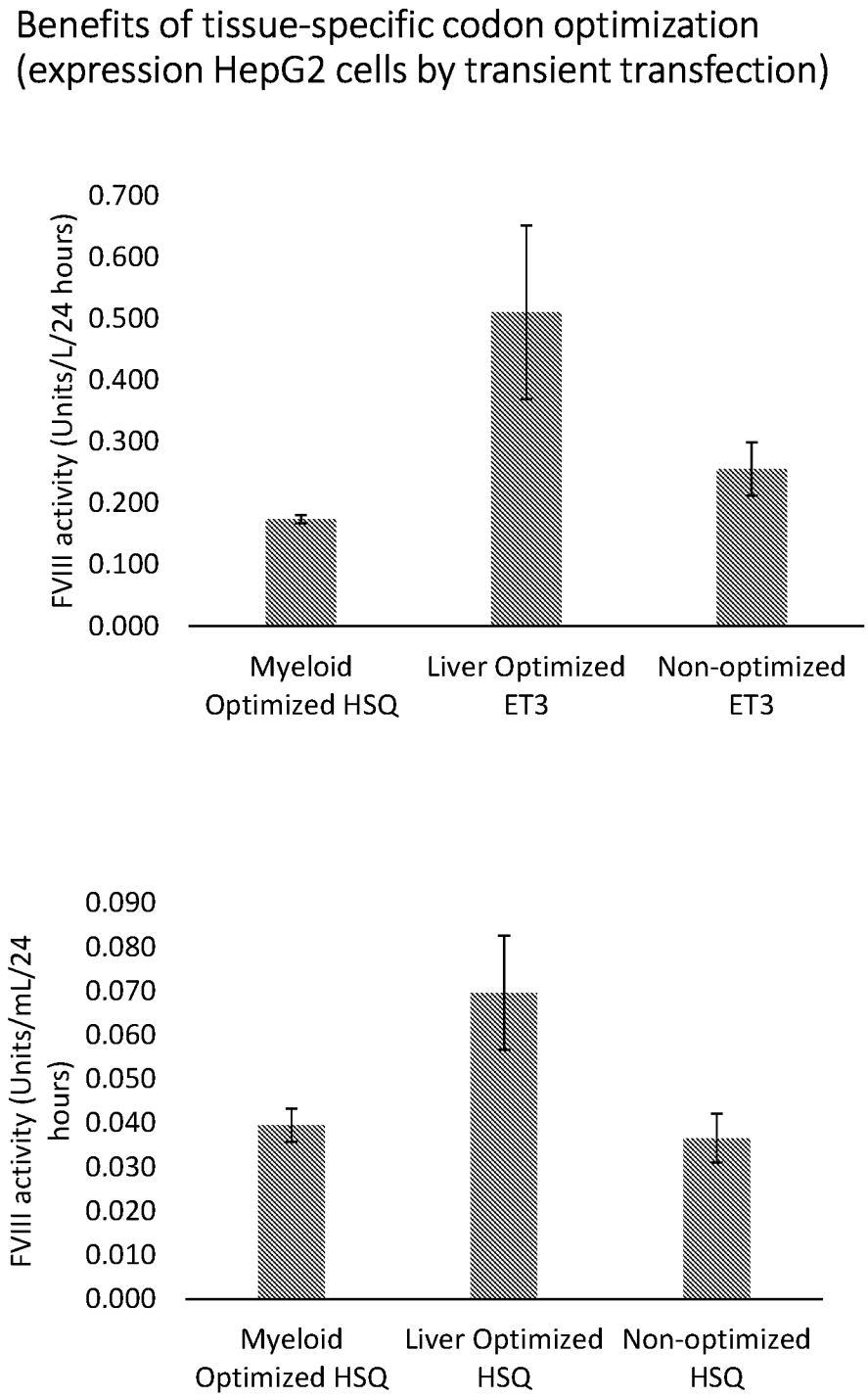

Benefits of tissue-specific codon optimization
(expression HepG2 cells by transient transfection)
FVIII activity (Units/L/24 hours)
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
Myeloid Optimized HSQ
Liver Optimized ET3
Non-optimized ET3
FVIII activity (Units/mL/24 hours)
0.090
0.080
0.070
0.060
0.050
0.040
0.030
0.020
0.010
0.000
Myeloid Optimized HSQ
Liver Optimized HSQ
Non-optimized HSQ

FIG. 5

Tissue-specific codon optimization expression in cells from different species (expression in baby hamster kidney cells by transient transfection)

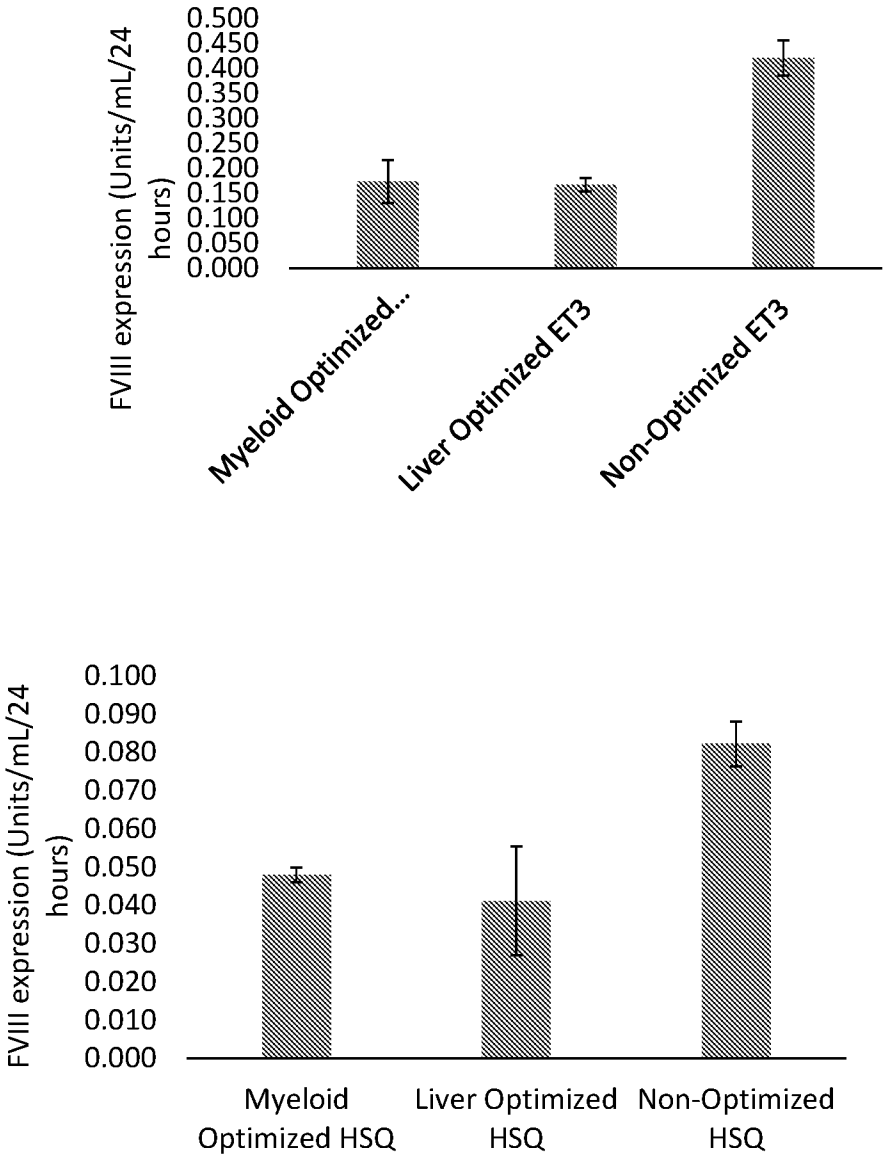


FIG. 6

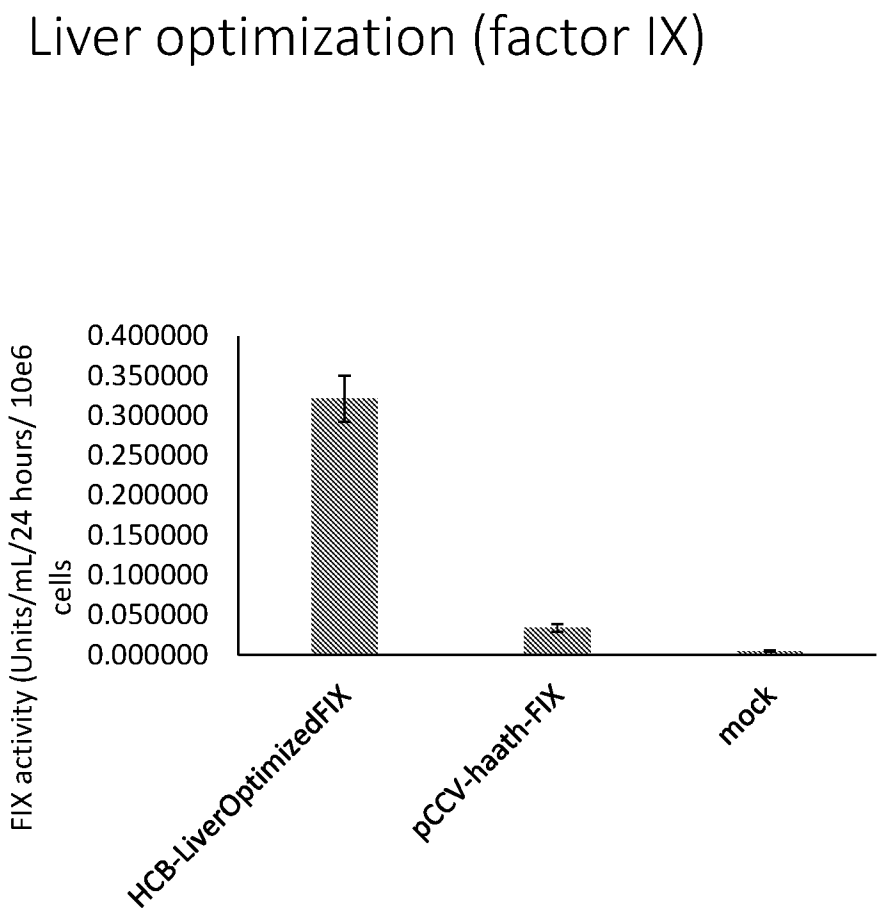

Liver optimization (factor IX)
FIX activity (Units/mL/24 hours/ 10e6 cells
0.400000
0.350000
0.300000
0.250000
0.200000
0.150000
0.100000
0.050000
0.000000
HCB-LiverOptimizedFIX
pCCV-haath-FIX
mock

FIG. 7

(1) ABP (SEQ ID NO: 113)

(2) (3)

GTTAATTTTTAAAAAGCAGTCAAAAGTCCAAGTGGCCCTTGCGAGCAT

TTACTCTCTCTGTTTGCTCTGGTTAATAATCTCAGGAGCACAAACA

(4) (5) (6)

(7) SynO (SEQ ID NO: 114)

GAGGTTAATAATTTTCCAGATCTCTCTGAGCAATAGTATAAAA

(8) (9)

(10) God (SEQ ID NO: 115)

AGTCATATGTTTGCTCACTGAAGGTTACTAGTTAACAGGCATCCCTTAAACAGGA

ABP-SynO (SEQ ID NO: 131)

(1) GTTAATTTTTAAAAAGCAGTCAAAAGTCCAAGTGGCCCTTGCGAGCATTTACTCTCTCTGTTTG
CTCTGGTTAATAATCTCAGGAGCACAAACA

(7) GAGGTTAATAATTTTCCAGATCTCTCTGAGCAATAGTATAAAA

ABP-Hp1-God (SEQ ID NO: 6)

(1) GTTAATTTTTAAAAAGCAGTCAAAAGTCCAAGTGGCCCTTGCGAGCATTTACTCTCTCTGTTTG
CTCTGGTTAATAATCTCAGGAGCACAAACA

GAG

(7) GTTAATAATTTTC

(10) AGTCATATGTTTGCTCACTGAAGGTTACTAGTTAACAGGCATCCCTTAAACAGGA

TATAAAA

(1) ABP element
(2) HNF1-1 TF binding site
(3) HNF4 TF binding site
(4) HNF3a TF binding site
(5) HNF1-2 TF binding site
(6) HNF3-2 TF binding site
(7) SynO element
(8) HP1 TF binding site
(9) TATA box
(10) God element

FIG. 8

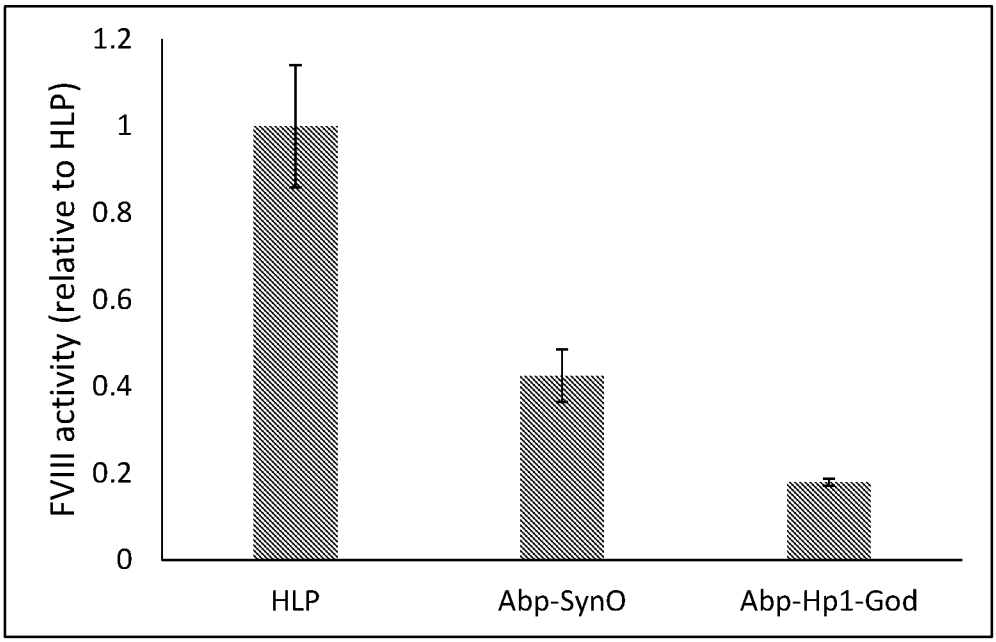

FVIII activity (relative to HLP)
1.2
1
0.8
0.6
0.4
0.2
0
HLP
Abp-SynO
Abp-Hp1-God

FIG. 9A

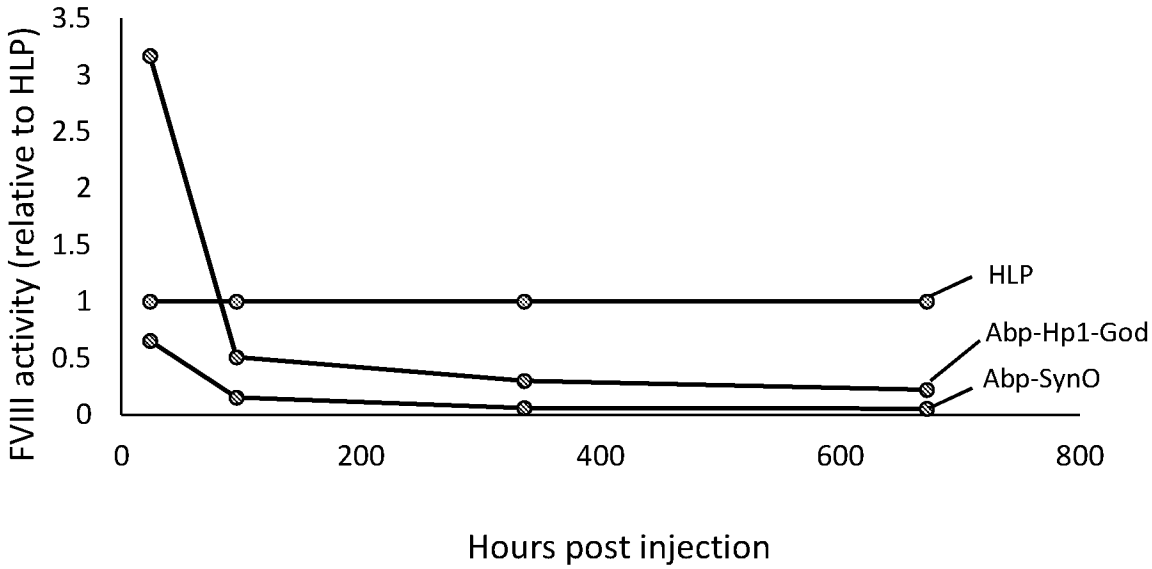

FVIII activity (relative to HLP)
3.5
3
2.5
2
1.5
1
0.5
0
0
200
400
600
800
HLP
Abp-Hp1-God
Abp-SynO
Hours post injection

FIG. 9B

(11) ABP-exact (SEQ ID NO: 116)

(12) (13)
GTTAATCATTAACTTAAAAAGCAGTCAAAAGTCCAAAGGTCAAAGGTCAGAGCATTTA
CTCTCTCCAATGTTGACTCTCGTTAATGATTAAGGAGCAATTGTTGACTT
(14) (15) (16)

(17) Short-ABP-exact (SEQ ID NO: 117)

(12) (13) (14) (15)
GTTAATCATTAACTTAGGTCAAAGGTCAGACAATGTTGACTCTCGTTAATGATTAACC
GCAATTGTTGACTT
(16)

(18) Transcription start site, TSS (nuc. 116-146 of SEQ ID NO: 4)

(19) (20)
GCCAGCAGCAGCCTGACCACATCTCATCCTC

(21) HNF1a TF binding site
GTTAATCATTAA

(22) Sp1 TF binding site
GTTAATCATTAA

(23) SV40 intron
CTCTAAGGTAAATATAAAATTTTTAAGTGTATAATGTGTTAAACTACTGATTCTAAT
TGTTTGTGTATTTTAGATTCCAACCTATGGAACTGA

(11) ABP-exact (consensus TF binding sites)
(12) HNF1-1 consensus TF binding site
(13) HNF4 consensus TF binding site
(14) HNF3a consensus TF binding site
(15) HNF1-2 consensus TF binding site
(16) HNF3-2 consensus TF binding site
(17) Short-ABP-exact
(18) Transcription Start Site (TSS)
(19) GC rich spacer
(20) Transcription start motif
(21) HNF1a TF binding site (liver-directed TF binding site)
(22) Sp1 TF binding site (liver-directed TF binding site)
(23) SV40 intron

FIG. 10

ABP-exact-SynO (SEQ ID NO: 118)

(11) GTTAATCATTAACTTAAAAAGCAGTCAAAAGTCCAAAGGTCAAAGGTCAGAGCATTTACTCTCTCCA
ATGTTGACTCTCGTTAATGATTAAGGAGCAATTGTTGACTT
(7) GAGGTTAATAATTTTCCAGATCTCTCTGAGCAATAGTATAAAA

ShortABP-exact-SynO (SEQ ID NO: 119)

(17) GTTAATCATTAACTTAGGTCAAAGGTCAGACAATGTTGACTCTCGTTAATGATTAACCGGAATTGTT
GACTT
(7) GAGGTTAATAATTTTCCAGATCTCTCTGAGCAATAGTATAAAA

ABP-HP1-God-TSS (SEQ ID NO: 7)

(1) GTTAATTTTTAAAAAGCAGTCAAAAGTCCAAGTGGCCCTTGCGAGCATTTACTCTCTCTGTTTG
CTCTGGTTAATAATCTCAGGAGCACAAACA
GAG
(8) GTTAATAATTTTC
(10) AGTCATATGTTTGCTCACTGAAGGTTACTAGTTAACAGGCATCCCTTAAACAGGA
TATAAAAG
(18) GCCAGCAGCAGCCTGACCACATCTCATCCTC

FIG. 11A

HNF1a-ABP-SynO (SEQ ID NO: 120)

(21) GTTAATCATTAA
GTC
(1) GTTAATTTTTAAAAAGCAGTCAAAAGTCCAAGTGGCCCTTGCGAGCATTTACTCTCTCTGTTTG
CTCTGGTTAATAATCTCAGGAGCACAAACA
(7) GAGGTTAATAATTTTCCAGATCTCTCTGAGCAATAGTATAAAA

Sp1-ABP-SynO (SEQ ID NO: 121)

(22) TGGGCGGAGT
GTC
(1) GTTAATTTTTAAAAAGCAGTCAAAAGTCCAAGTGGCCCTTGCGAGCATTTACTCTCTCTGTTTG
CTCTGGTTAATAATCTCAGGAGCACAAACA
(7) GAGGTTAATAATTTTCCAGATCTCTCTGAGCAATAGTATAAAA

HNF1-ShortABPExact-SynO-TSS-Int (SEQ ID NO: 112)

(21) GTTAATCATTAA
GTC
(17) GTTAATCATTAACTTAGGTCAAAGGTCAGACAATGTTGACTCTCGTTAATGATTAACCGGAATTGT
TGACTT
(7) GAGGTTAATAATTTTCCAGATCTCTCTGAGCAATAGTATAA
AAG
(18) GCCAGCAGCAGCCTGACCACATCTCATCCTC
(23) CTCTAAGGTAAATATAAAATTTTTAAGTGTATAATGTGTTAAACTACTGATTCTAATTGTTTGTGT
ATTTTAGATTCCAACCTATGGAACTGA

FIG. 11B

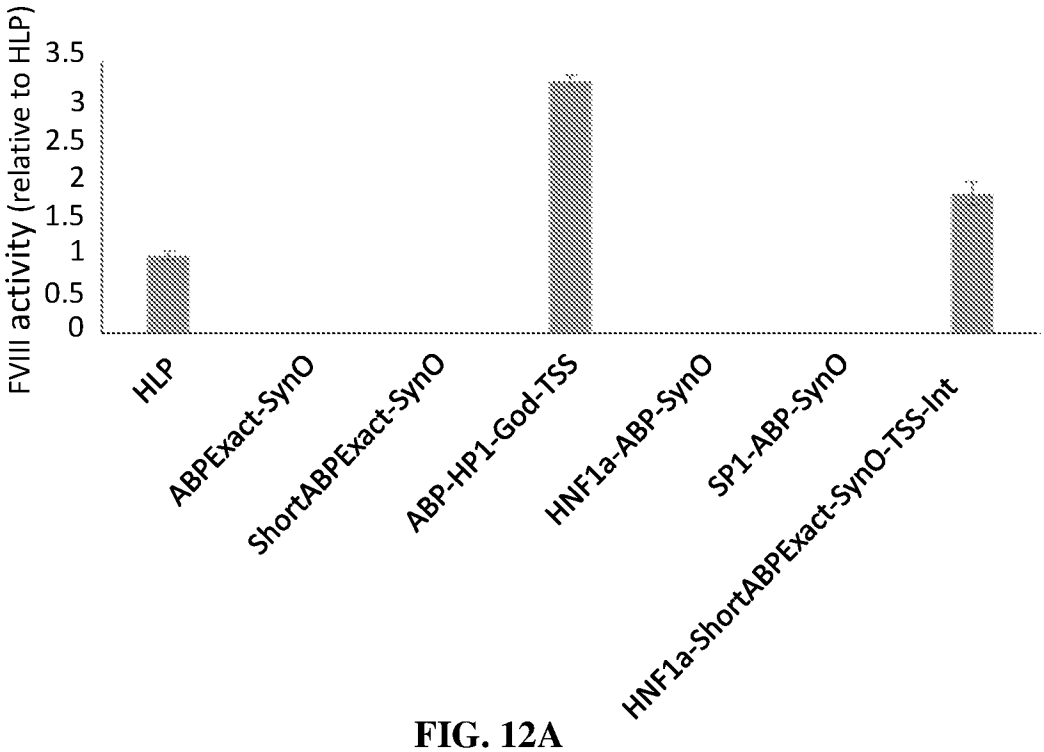


FIG. 12A

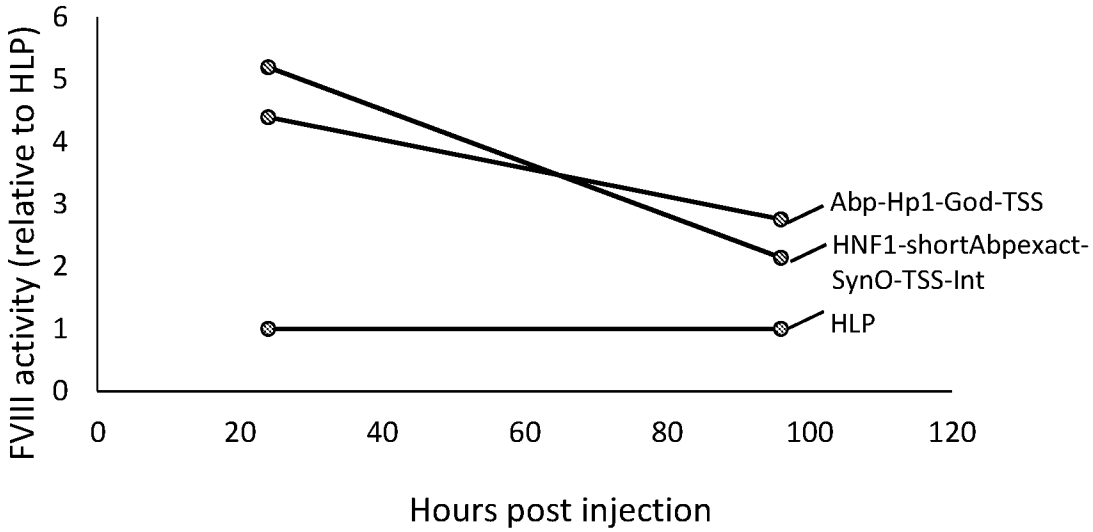


FIG. 12B

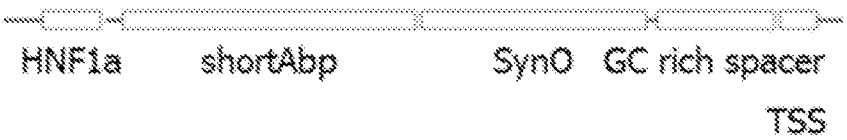
(24) shortABP (SEQ ID NO: 34)

GTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACA

(2) (3) (4) (5) (6)

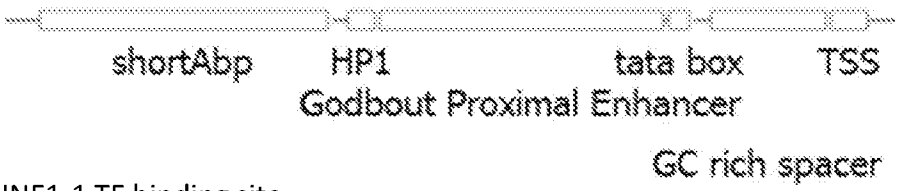
(25) HNF1-shortABP-SynO-TSS (also called Hepatic Combinatorial Bundle, or HCB) (SEQ ID NO: 4)

(21) GTTAATCATTAA
GTC
(24) GTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACA
(7) GAGGTTAATAATTTTCCAGATCTCTCTGAGCAATAGTATAAAA
G
(18) GCCAGCAGCAGCCTGACCACATCTCATCCTC



(27) shortABP-HP1-God-TSS (SEQ ID NO: 5)

(24) GTTAATTTTTGTGGCCCTTGCGATGTTTGCTCTGGTTAATAATCTCAGGACAAACA
TAC
(8a) ATTTTC
(10) AGTCATATGTTTGCTCACTGAAGGTTACTAGTTAACAGGCATCCCTTAAACAGGA
TATAAAAG
(18) GCCAGCAGCAGCCTGACCACATCTCATCCTC



(2) HNF1-1 TF binding site
(3) HNF4 TF binding site
(4) HNF3a TF binding site
(5) HNF1-2 TF binding site
(6) HNF3-2 TF binding site
(7) SynO element
(8a) shortened HP1 TF binding site
(10) God element
(18) Transcription Start Site (TSS)
(21) HNF1a TF binding site
(24) shortABP
(25) HNF1-shortABP-SynO-TSS (Hepatic Combinatorial Bundle, or HCB)
(27) shortABP-HP1-God-TSS

FIG. 13

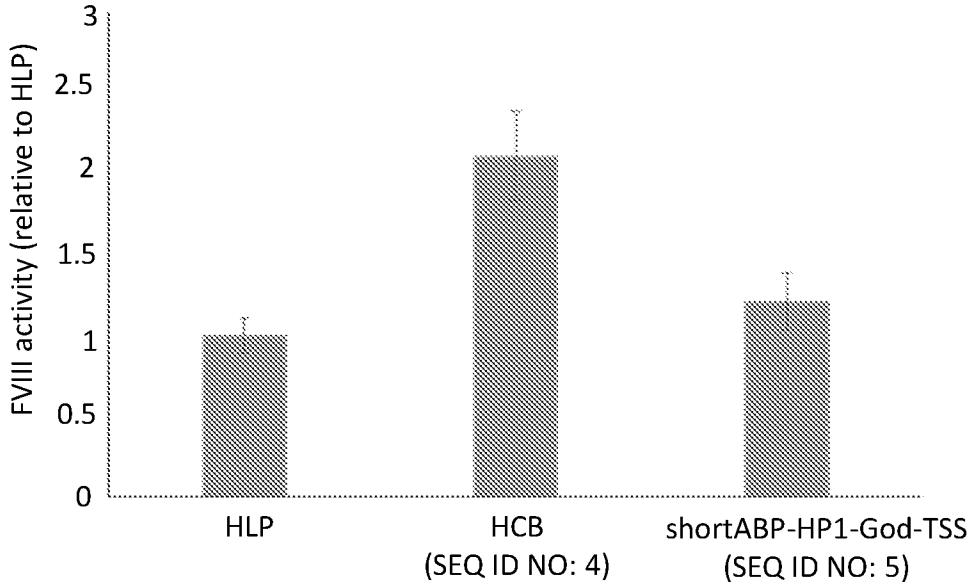


FIG. 14A

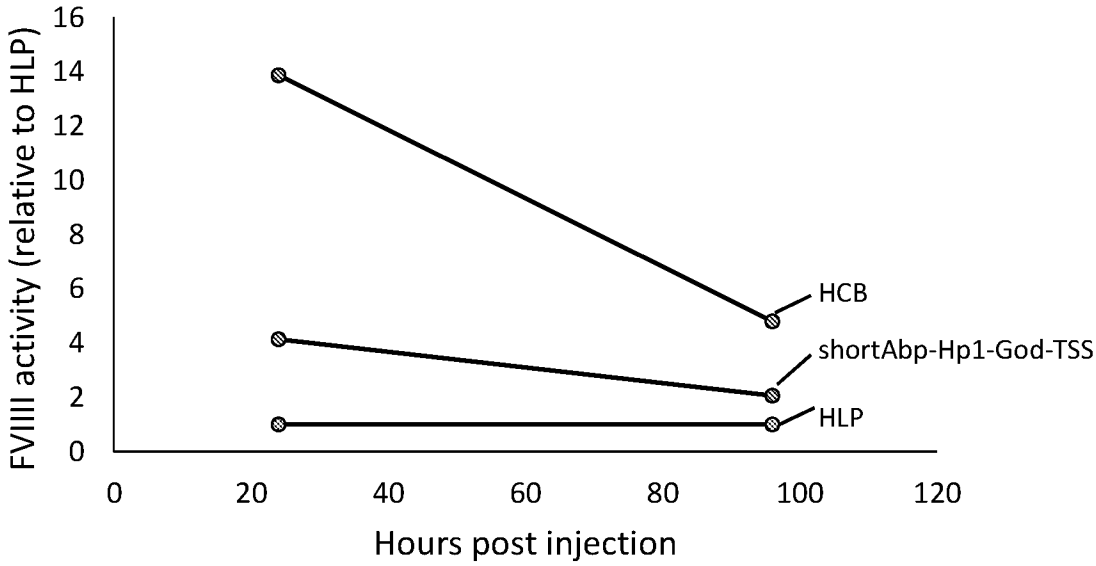


FIG. 14B

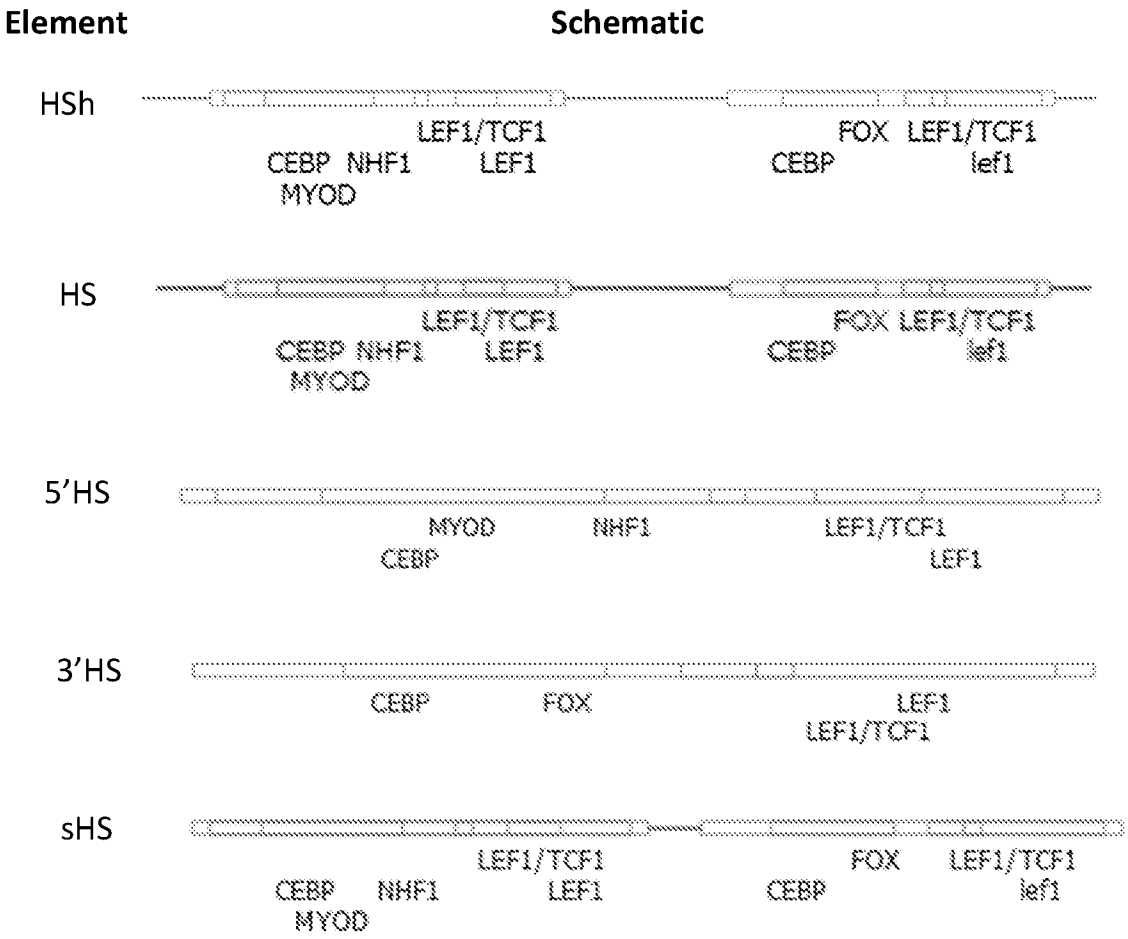

Element
Schematic
HSh
LEF1/TCF1
CEBP NHF1 LEF1
MYOD
FOX LEF1/TCF1
CEBP lef1
HS
LEF1/TCF1
CEBP NHF1 LEF1
MYOD
FOX LEF1/TCF1
CEBP lef1
5'HS
MYOD NHF1 LEF1/TCF1
CEBP LEF1
3'HS
CEBP FOX LEF1
LEF1/TCF1
sHS
LEF1/TCF1
CEBP NHF1 LEF1
MYOD
FOX LEF1/TCF1
CEBP lef1

FIG. 15

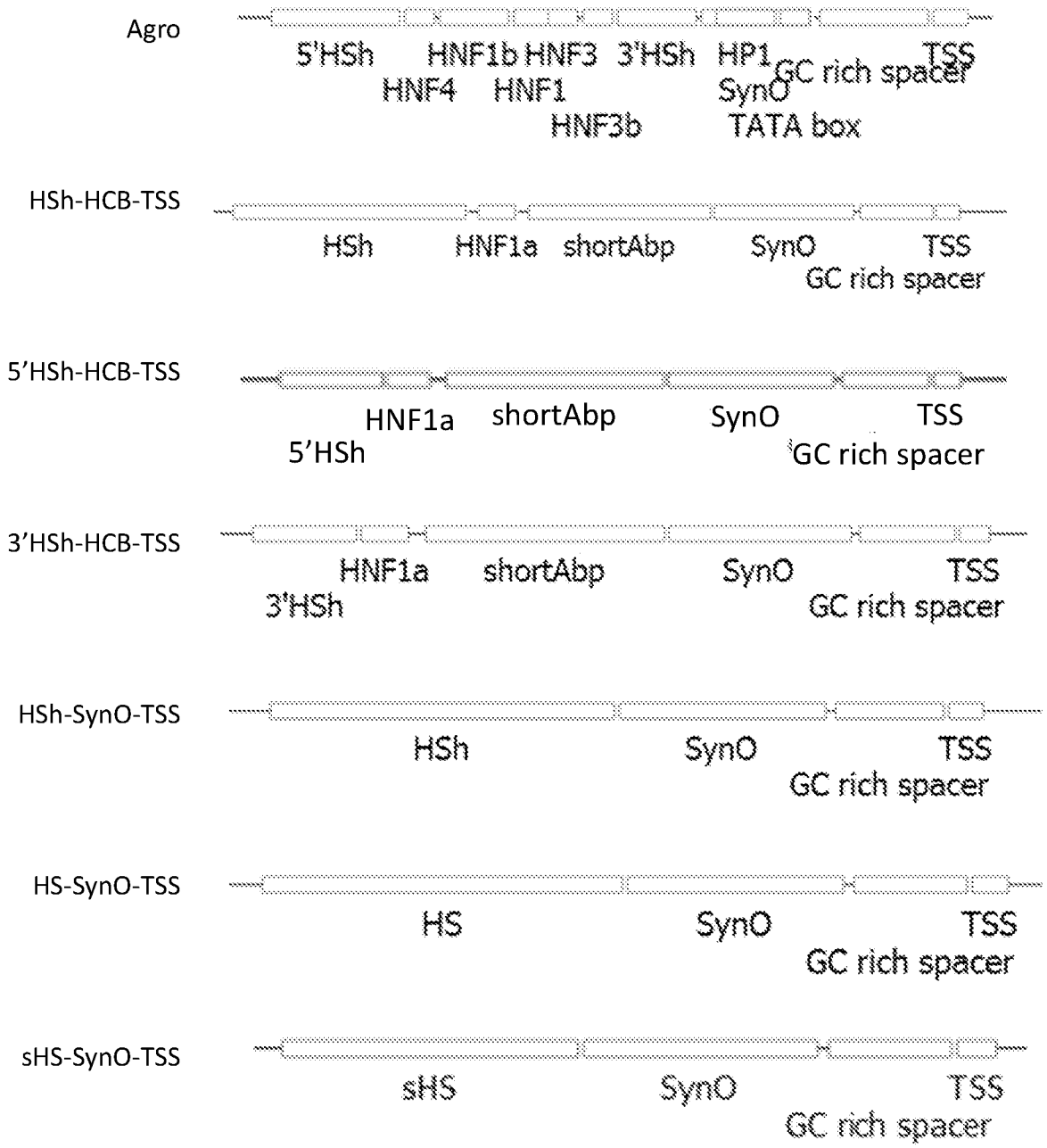

Agro
5'HSh
HNF1b
HNF3
3'HSh
HP1
TSS
GC rich spacer
HNF4
HNF1
SynO
HNF3b
TATA box
HSh-HCB-TSS
HSh
HNF1a
shortAbp
SynO
TSS
GC rich spacer
5'HSh-HCB-TSS
HNF1a
shortAbp
SynO
TSS
5'HSh
GC rich spacer
3'HSh-HCB-TSS
HNF1a
shortAbp
SynO
TSS
3'HSh
GC rich spacer
HSh-SynO-TSS
HSh
SynO
TSS
GC rich spacer
HS-SynO-TSS
HS
SynO
TSS
GC rich spacer
sHS-SynO-TSS
sHS
SynO
TSS
GC rich spacer

FIG. 16

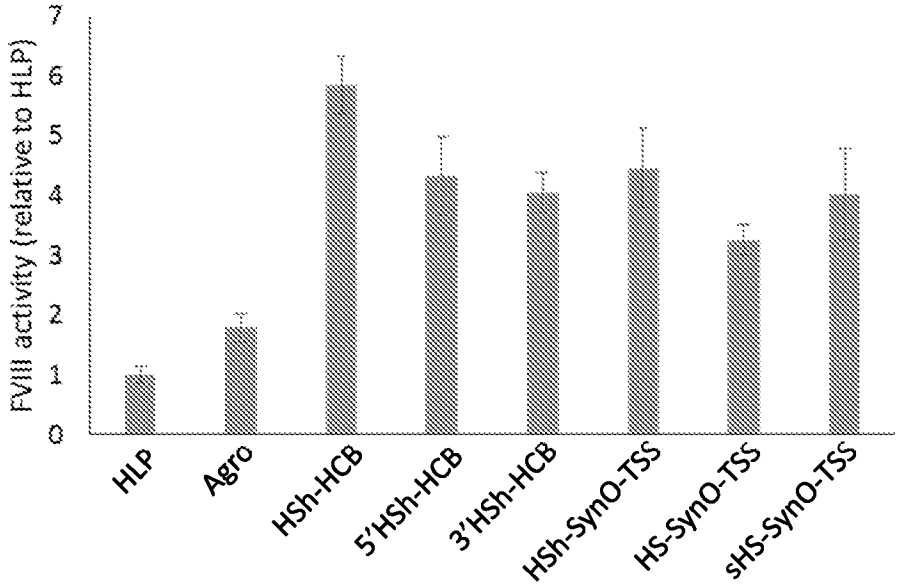

FVIII activity (relative to HLP)
0
1
2
3
4
5
6
7
HLP
Agro
HSh-HCB
5'HSh-HCB
3'HSh-HCB
HSh-SynO-TSS
HS-SynO-TSS
sHS-SynO-TSS

FIG. 17

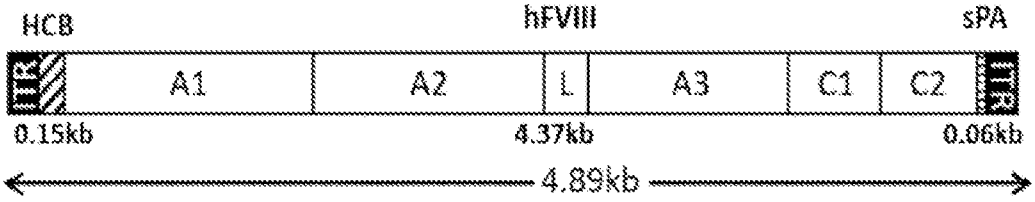

HCB
hFVIII
sPA
A1
A2
L
A3
C1
C2
0.15kb
4.37kb
0.06kb
4.89kb

FIG. 18

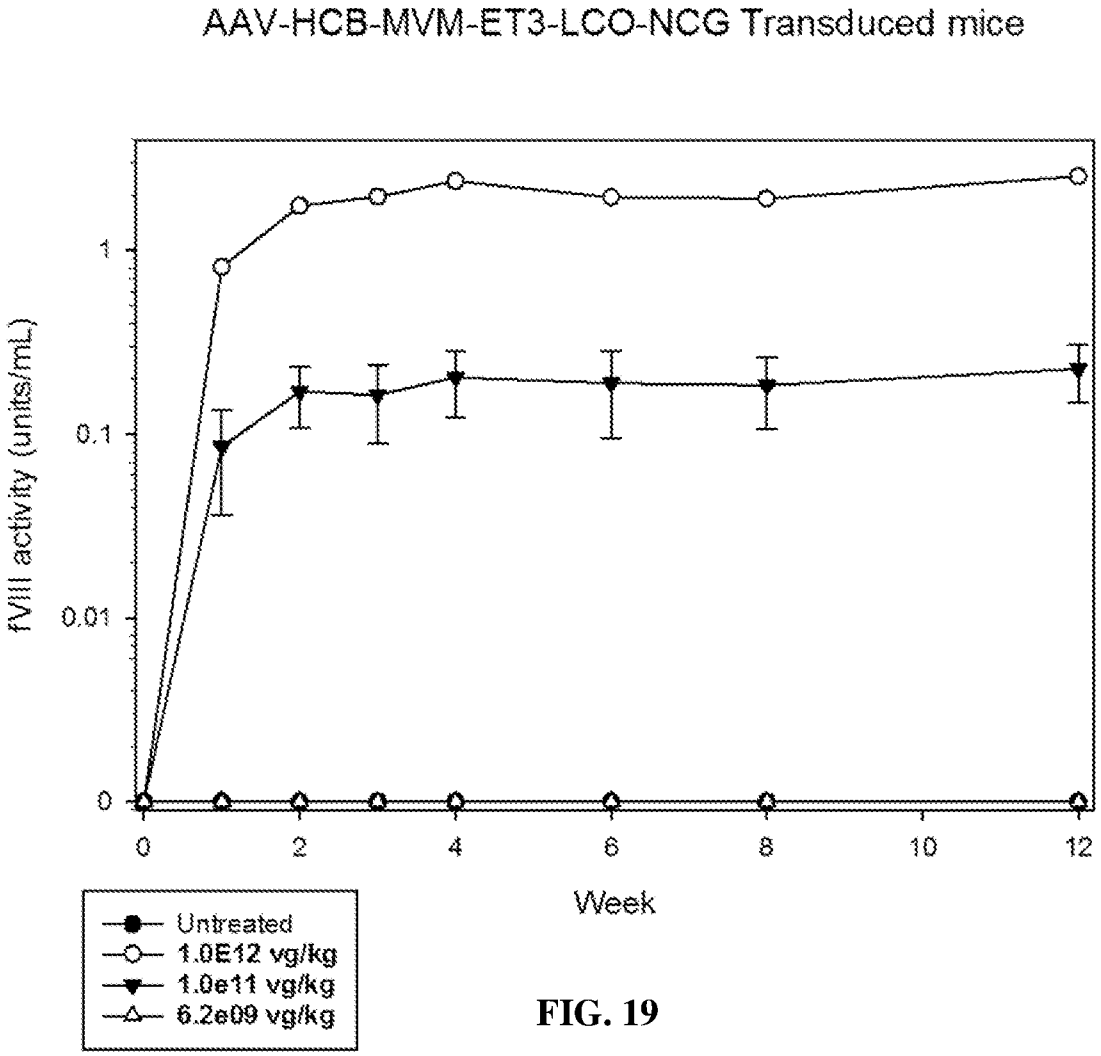

AAV-HCB-MVM-ET3-LCO-NCG Transduced mice
fVIII activity (units/mL)
1
0.1
0.01
0
0
2
4
6
8
10
12
Week
Untreated
1.0E12 vg/kg
1.0e11 vg/kg
6.2e09 vg/kg

FIG. 19