

[0009] Accordingly, use of adeno-associated virus (AAV) vectors for gene therapy is limited due to the single administration to patients (owing to the patient immune response), the limited range of transgene genetic material suitable for delivery in AAV vectors due to minimal viral packaging capacity (about 4.5kb), and slow AAV-mediated gene expression.

[0010] There is large unmet need for disease-modifying therapies in hemophilia A. Current therapies are burdensome and require, *e.g.*, slow drip intravenous (IV) administrations. First, these Factor VIII injectables do not provide continuous delivery of factors, with trough levels allowing bleeding episodes. Second, there are no approved gene therapies for hemophilia A, and AAV based therapies cannot be used by 25% to 40% of patients due to pre-existing antibodies. AAV can only be administered once, and the resulting Factor VIII levels might not reach clinical significance, or may be supranormal, as dose levels cannot be titrated. Third, many hemophilia A patients cannot utilize these therapies because of the development of neutralizing antibodies to these exogenous, artificial clotting factors.

[0011] Accordingly, there is need in the field for a technology that permits expression of a therapeutic FVIII protein in a cell, tissue or subject for the treatment of hemophilia A.

SUMMARY

[0012] The technology described herein relates to methods and compositions for treatment of hemophilia A by expression of Factor VIII (FVIII) protein from a capsid-free (*e.g.*, non-viral) DNA vector with covalently-closed ends (referred to herein as a “closed-ended DNA vector” or a “ceDNA vector”), where the ceDNA vector comprises a FVIII nucleic acid sequence or codon optimized versions thereof. These ceDNA vector can be used to produce FVIII proteins for treatment, monitoring, and diagnosis. The application of ceDNA vectors expressing FVIII to the subject for the treatment of hemophilia A is useful to: (i) provide disease modifying levels of FVIII enzyme, (ii) be minimally invasive in delivery, (iii) be repeatable and dosed-to-effect, (iv) have rapid onset of therapeutic effect, (v) result in sustained expression of corrective FVIII enzyme in the liver, (vi) restore urea cycle function, and/or (vii) be titratable to achieve the appropriate pharmacologic levels of the defective enzyme.

[0013] In examples, a ceDNA-vector expressing FVIII is optionally present in a liposome nanoparticle formulation (LNP) for the treatment of hemophilia A. The ceDNA vectors described herein can provide one or more benefits including, but not limited to providing disease modifying levels of FVIII, being minimally invasive in delivery, being repeatable and dosed-to-effect, providing a rapid onset of therapeutic effect, *e.g.*, in some examples, within days of therapeutic intervention, providing sustained expression of corrective Factor VIII levels in the circulation, being titratable to achieve the appropriate pharmacologic levels of the defective coagulation factor, and/or providing treatments for other types of hemophilia, including but not limited to Factor VIII deficiency (hemophilia A) or Factor IX deficiency (hemophilia B) or Factor XI deficiency (hemophilia C).

[0014] Accordingly, the disclosure described herein relates to a capsid-free (*e.g.*, non-viral) DNA vector with covalently-closed ends (referred to herein as a “closed-ended DNA vector” or a “ceDNA vector”) comprising a heterogeneous gene encoding FVIII, to permit expression of the FVIII therapeutic protein in a cell (*e.g.*, hepatocytes of a human patient suffering from hemophilia A).

[0015] According to one aspect, the disclosure provides a capsid-free close-ended DNA (ceDNA) vector comprising: at least one nucleic acid sequence between flanking inverted terminal repeats (ITRs), wherein at least one nucleic acid sequence encodes at least one Factor VIII (FVIII) protein, wherein the at least one nucleic acid sequence has at least 85% identity to a sequence selected from the group consisting of SEQ ID NOs: 76, 71-75, 77-183, 556 and 626-633. In one example, the present disclosure provides a capsid-free close-ended DNA (ceDNA) vector comprising at least one nucleic acid sequence, *e.g.*, heterologous nucleic acid sequence, between flanking inverted terminal repeats (ITRs), wherein at least one heterologous nucleic acid sequence encodes at least one FVIII protein, wherein the at least one nucleic acid sequence that encodes at least one FVIII protein is selected from any of the sequences in **Table 1A** (SEQ ID NOs: 71-183, 556 and 626-633).

[0016] In a first example, the disclosure provides a capsid-free close-ended DNA (ceDNA) vector comprising at least one nucleic acid sequence between flanking inverted terminal repeats (ITRs), wherein the at least one nucleic acid sequence encodes at least one FVIII protein, wherein the at least one nucleic acid sequence that encodes at least one FVIII protein is selected from a sequence having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or least 99% identity to any sequence in **Table 1A** (SEQ ID NOs: 71-183, 556 and 626-633). According to some examples, the at least one nucleic acid sequence that encodes at least one FVIII protein is at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or least 99% identical to SEQ ID NO: 556. According to some examples, the at least one nucleic acid sequence that encodes at least one FVIII protein consists of SEQ ID NO: 556. According to some examples, the at least one nucleic acid that encodes at least one FVIII protein comprises SEQ ID NO: 556, wherein SEQ ID NO: 556 further comprises one or more modifications. According to some examples, the at least one nucleic acid comprising SEQ ID NO: 556, further comprising one or more modifications comprises or consists of SEQ ID NO: 627. According to some examples, the at least one nucleic acid comprising SEQ ID NO: 556, further comprising one or more modifications comprises or consists of SEQ ID NO: 628. According to some examples, the at least one nucleic acid comprising SEQ ID NO: 556, further comprising one or more modifications comprises or consists of SEQ ID NO: 628. According to some examples, the at least one nucleic acid comprising SEQ ID NO: 556, further comprising one or more modifications comprises or consists of SEQ ID NO: 630. According to some examples, the at least one nucleic acid comprising SEQ ID NO: 556, further comprising one or more modifications comprises or consists of SEQ ID NO: 631. According to some examples, the at least one nucleic acid comprising SEQ ID NO: 556, further comprising one or more modifications comprises or consists of SEQ ID NO: 632. According to some examples, the at least one nucleic acid comprising SEQ ID NO: 556, further comprising one or more modifications comprises or consists of SEQ ID NO: 633.

[0017] In some examples, the ceDNA vector comprises a promoter or promoter set operatively linked to the least one nucleic acid sequence that encodes at least one FVIII protein. According to some examples, the at least one nucleic acid sequence that encodes at least one FVIII protein is selected from any of the sequences in **Table 1A** (SEQ ID NOs: 71-183, 556 and 626-633). In some examples, the ceDNA vector comprises a promoter selected from the group consisting of human $\alpha 1$ antitrypsin (hAAT) promoter, minimal transthyretin promoter (TTRm), hAAT_core_C06, hAAT_core_C07, hAAT_core_08, hAAT_core_C09, hAAT_core_C10, and hAAT_core_truncated. In some examples, the ceDNA vector comprises a promoter selected from a nucleic acid sequence having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or least 99% identity to any one of SEQ ID NOs: 210-217. In some examples, the promoter set comprises a synthetic liver specific promoter set including enhancers and a core promoter, without a 5pUTR. In some examples, the promoter set is selected from a nucleic acid sequence having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or least 99% identity to any one of SEQ ID NOs: 184-197, 400, 401, 484, and 617-624.

[0018] According to some examples, the at least one nucleic acid sequence that encodes the at least one FVIII protein is selected from any of the sequences in **Table 1A** (SEQ ID NOs: 71-183, 556 and 626-633) and the promoter set is selected from a nucleic acid sequence having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or least 99% identity to any one of SEQ ID NOs: 184-197, 400, 401, 484, and 617-624. According to some examples, the at least one nucleic acid sequence that encodes the at least one FVIII protein is selected from a nucleic acid sequence having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or least 99% identity to any one of SEQ ID NOs: 556 or 626-633 and the promoter set is selected from a nucleic acid sequence having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or least 99% identity to any one of SEQ ID NOs: 184-197, 400, 401, 484, and 617-624.

[0019] In some examples, the ceDNA vector comprises an enhancer. In some examples, the enhancer is selected from the group consisting of: a Serpin enhancer (SerpEnh), the transthyretin (TTRe) gene enhancer (TTRe), the Hepatic Nuclear Factor 1 binding site (HNF1), Hepatic Nuclear Factor 4 binding site (HNF4), Human apolipoprotein E/C-I liver specific enhancer (ApoE_Enh), the enhancer region from Pro-albumin gene (ProEnh), a CpG minimized version of the ApoE_Enh (Human apolipoprotein E/C-I liver specific enhancer) (ApoE_Enh_C03, ApoE_Enh_C04, ApoE_Enh_C09, and ApoE_Enh_C10), and Hepatic nuclear factor enhancer array embedded in GE-856 (Embedded_enhancer_HNF_array). In some examples, the Serpin enhancer comprises a nucleic acid sequence at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or least 99% identical to SEQ ID NO: 198. In some examples, the enhancer is selected from a nucleic acid sequence set forth in **Table 7** (SEQ ID NOs: 198-209, 485 and 557-616). In some examples, the enhancer is selected from a nucleic acid sequence having at least 85%, at least 90%,

at least 95%, at least 96%, at least 97%, at least 98%, or least 99% identity to any sequence in **Table 7** (SEQ ID NOs: 198-209, 485 and 557-616).

[0020] According to some examples, the at least one nucleic acid sequence that encodes the at least one FVIII protein is selected from any of the sequences in **Table 1A** (SEQ ID NOs: 71-183, 556 and 626-633) and the enhancer is selected from a nucleic acid sequence having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or least 99% identity to any one of SEQ ID NOs: 198-209, 485 and 557-616. According to some examples, the at least one nucleic acid sequence that encodes the at least one FVIII protein is selected from a nucleic acid sequence having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or least 99% identity to any one of SEQ ID NOs: 556 or 626-633 and the enhancer is selected from a nucleic acid sequence having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or least 99% identity to any one of SEQ ID NOs: 557-616.

[0021] In some examples, the ceDNA vector comprises a 5' UTR sequence. In some examples, the 5' UTR sequence is selected from a sequence having at least 85% identity to any sequence in **Table 10**. In some examples, the ceDNA vector comprises an intron sequence. In some examples, the intron sequence is selected from a sequence having at least 85% identity to any sequence in **Table 11**. In some examples, the ceDNA vector comprises an exon sequence. In some examples, the exon sequence is selected from a sequence having at least 85% identity to any sequence in **Table 12**. In some examples, the ceDNA vector comprises a 3' UTR sequence. In some examples, the exon sequence is selected from a sequence having at least 85% identity to any sequence in **Table 13**. In some examples, the ceDNA vector comprises at least one poly A sequence. In some examples, the ceDNA vector comprises one or more DNA nuclear targeting sequences (DTS). In some examples, the DTS is selected from a sequence having at least 85% identity to any sequence in **Table 14**. In some examples, the ceDNA vector comprises one or more of the following Ubiquitous Chromatin-opening Elements (UCOE)s, Kozak sequences, spacer sequences or leader sequences.

[0022] In one example of any of the foregoing aspects of examples, at least one nucleic acid sequence is cDNA.

[0023] In one example of any of the foregoing aspects of examples, at least one ITR comprises a functional terminal resolution site and a Rep binding site.

[0024] In one example of any of the foregoing aspects of examples, one or both of the ITRs are from a virus selected from a parvovirus, a dependovirus, and an adeno-associated virus (AAV). In some examples, the flanking ITRs are symmetric or asymmetric. In some examples, the flanking ITRs are symmetrical or substantially symmetrical. In some examples, the flanking ITRs are asymmetric. In some examples, one or both of the ITRs are wild-type, or wherein both of the ITRs are wild-type. In some examples, the flanking ITRs are from different viral serotypes. In

some examples, the flanking ITRs are from the same viral serotypes. In some examples, the flanking ITRs are from a pair of viral serotypes shown in Table 6 of International Publication No. WO/2019/161059 (incorporated by reference in its entirety herein). In some examples, one or both of the ITRs comprises a sequence selected from the sequences in **Table 2, Table 4A, Table 4B, or Table 5**. In some examples, at least one of the ITRs is altered from a wild-type AAV ITR sequence by a deletion, addition, or substitution that affects the overall three-dimensional conformation of the ITR. In some examples, one or both of the ITRs are derived from an AAV serotype selected from AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, and AAV12. In some examples, one or both of the ITRs are synthetic. In some examples, one or both of the ITRs is not a wild-type ITR, or wherein both of the ITRs are not wild-type. In some examples, one or both of the ITRs is modified by a deletion, insertion, and/or substitution in at least one of the ITR regions selected from A, A', B, B', C, C', D, and D'. In some examples, the deletion, insertion, and/or substitution results in the deletion of all or part of a stem-loop structure normally formed by the A, A', B, B' C, or C' regions. In some examples, one or both of the ITRs are modified by a deletion, insertion, and/or substitution that results in the deletion of all or part of a stem-loop structure normally formed by the B and B' regions. In some examples, one or both of the ITRs are modified by a deletion, insertion, and/or substitution that results in the deletion of all or part of a stem-loop structure normally formed by the C and C' regions. In some examples, one or both of the ITRs are modified by a deletion, insertion, and/or substitution that results in the deletion of part of a stem-loop structure normally formed by the B and B' regions and/or part of a stem-loop structure normally formed by the C and C' regions. In some examples, one or both of the ITRs comprise a single stem-loop structure in the region that normally comprises a first stem-loop structure formed by the B and B' regions and a second stem-loop structure formed by the C and C' regions. In some examples, one or both of the ITRs comprise a single stem and two loops in the region that normally comprises a first stem-loop structure formed by the B and B' regions and a second stem-loop structure formed by the C and C' regions. In some examples, one or both of the ITRs comprise a single stem and a single loop in the region that normally comprises a first stem-loop structure formed by the B and B' regions and a second stem-loop structure formed by the C and C' regions. In some examples, both ITRs are altered in a manner that results in an overall three-dimensional symmetry when the ITRs are inverted relative to each other. In some examples, one or both of the ITRs comprises a nucleic acid sequence selected from the sequences in **Tables 2, 4A, 4B, and 5**.

[0025] In some examples of any of the above aspects or examples, the ceDNA vector comprises a nucleic acid sequence selected from a sequence having at least 85% identity, at least 90% identity, 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 at least 100% identity with a sequence in **Table 18** (e.g., any one of SEQ ID NOs: 1-70, 442-483, or 642-646).

[0026] In another aspect, the disclosure provides the use of the ceDNA vector as described herein in the manufacture of a medicament for expressing an FVIII protein in a cell. In one example, the disclosure provides a method of expressing an FVIII protein in a cell comprising contacting the cell with the ceDNA vector of any one of the aspects or examples herein. In some examples, the cell is a photoreceptor or a RPE cell. In some examples, the cell is *in vitro* or *in vivo*. In some examples of any of the above aspects or examples, the at least one nucleic acid sequence is codon optimized for expression in the eukaryotic cell. In some examples of any of the above aspects or examples, the at least one nucleic acid sequence is a sequence having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or least 99% identity to any sequence set forth in **Table 1A** (e.g., any one of SEQ ID NOs: 71-183, 556 and 626-633).

[0027] In another aspect, the disclosure provides the use of the ceDNA vector as described herein in the manufacture of a medicament for treating a subject with hemophilia A. In one example, the disclosure provides a method of treating a subject with hemophilia A, comprising administering to the subject a ceDNA vector of any one of the aspects or examples herein, wherein at least one nucleic acid sequence encodes at least one FVIII protein.

[0028] In another aspect, the disclosure provides the use of a nucleic acid sequence having at least 85% identity with a sequence selected from the group consisting of any of the sequences set forth in **Table 18** in the manufacture of a medicament for treating a subject with hemophilia A. In one example, the disclosure provides a method of treating a subject with hemophilia A, comprising administering to the subject a nucleic acid sequence selected from a sequence having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or least 99% identity with a sequence in **Table 18** (e.g., any one of SEQ ID NOs: 1-70, 442-483, or 642-646). According to one example, the nucleic acid sequence is at least 85% identical, at least 90% identical, at least 91% identical, at least 92% identical, at least 93% identical, at least 94% identical, at least 95% identical, at least 96% identical, at least 96% identical, at least 97% identical, at least 98% identical or at least 99% identical to SEQ ID NO: 5. In one example, the nucleic acid sequence comprises SEQ ID NO: 5. In another example, the nucleic acid sequence consists of SEQ ID NO: 5. In some examples of any of the above aspects or examples, the ceDNA vector comprises a nucleic acid sequence selected from a sequence having at least 85% identity, at least 90% identity, 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 100% identity with SEQ ID NO: 42. In some examples, the ceDNA comprises a nucleic acid sequence consisting of SEQ ID NO: 42.

[0029] In another example, the disclosure provides a method of treating a subject with hemophilia B, comprising administering to the subject a nucleic acid sequence selected from a sequence having at least 85% identity, at least 90% identity, 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 at least 100% identity with a sequence in **Table 18** (e.g., any one of SEQ ID NOs: 1-70, 442-483, or 642-646).

[0030] In some examples of any of the above aspects or examples, levels of FVIII in the serum of the subject are increased in subjects administered the ceDNA vector compared to a control. In some examples, the increase in levels of FVIII is greater than about 40% compared

to the control. In some examples, the at least one nucleic acid sequence is a sequence having at least 85% identity, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or least 99% to any sequence set forth in **Table 1A** (e.g., any one of SEQ ID NOs: 71-183, 556 and 626-633) or **Table 18** (e.g., any one of SEQ ID NOs: 1-70, 442-483, or 642-646). According to one example, the nucleic acid sequence is at least 85% identical, at least 90% identical, at least 91% identical, at least 92% identical, at least 93% identical, at least 94% identical, at least 95% identical, at least 96% identical, at least 96% identical, at least 97% identical, at least 98% identical or at least 99% identical to SEQ ID NO: 5. According to one example, the nucleic acid sequence comprises SEQ ID NO:5 or consists of SEQ ID NO: 5.

[0031] In some examples of the foregoing aspect or examples, a level of FVIII in the plasma of the subject is increased in the subject after administration. In some examples, the level of FVIII in the plasma of the subject is increased by at least about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 2-fold, 2.5-fold, 3-fold, 3.5-fold, 4-fold, 5-fold, 10-fold, 15-fold, 20-fold, 25-fold, 30-fold, 40-fold, 50-fold, 60-fold, 70-fold, 80-fold, 90-fold, or 100-fold after administration. In some examples, a level of FVIII in the serum of the subject is increased in the subject administered the ceDNA vector as compared to a control. In some examples, the increase in the level of FVIII in the serum of the subject is greater than about 40%, 50%, 60%, 70%, 80%, 90%, 100%, 2-fold, 2.5-fold, 3-fold, 3.5-fold, 4-fold, 5-fold, 10-fold, 15-fold, 20-fold, 25-fold, 30-fold, 40-fold, 50-fold, 60-fold, 70-fold, 80-fold, 90-fold, or 100-fold compared to the control. In some examples, the control is a level of FVIII in the serum of the subject prior to administration, or wherein the control is a level of FVIII in the serum of a subject having hemophilia A who did not receive the administration.

[0032] In some examples, the ceDNA vector is administered at a dose of about 0.1 mg/kg, 0.2 mg/kg, 0.3 mg/kg, 0.4 mg/kg, 0.5 mg/kg, 0.75 mg/kg, 1 mg/kg, 1.5 mg/kg, 2 mg/kg, 2.5 mg/kg, 3 mg/kg, 3.5 mg/kg, 4 mg/kg, 5 mg/kg, 6 mg/kg, 7 mg/kg, 8 mg/kg, 9 mg/kg, or 10 mg/kg. In some examples, the ceDNA vector is administered at a dose of about 0.1 mg/kg to about 20 mg/kg. In some examples, the ceDNA vector is administered at a dose of about 0.1 mg/kg to about 15 mg/kg. In some examples, the ceDNA vector is administered at a dose of about 0.1 mg/kg to about 10 mg/kg. In some examples, the ceDNA vector is administered at a dose of about 0.1 mg/kg to about 5 mg/kg. In some examples, the ceDNA vector is administered at a dose of about 0.1 mg/kg to about 0.5 mg/kg. In some examples, the ceDNA vector is administered at a dose of about 0.5 mg/kg to about 20 mg/kg. In some examples, the ceDNA vector is administered at a dose of about 0.5 mg/kg to about 15 mg/kg. In some examples, the ceDNA vector is administered at a dose of about 0.5 mg/kg to about 10 mg/kg. In some examples, the ceDNA vector is administered at a dose of about 0.5 mg/kg to about 5 mg/kg. In some examples, the ceDNA vector is administered at a dose of about 1 mg/kg to about 20 mg/kg. In some examples, the ceDNA vector is administered at a dose of about 1 mg/kg to about 15 mg/kg. In some examples, the ceDNA vector

is administered at a dose of about 1 mg/kg to about 10 mg/kg. In some examples, the ceDNA vector is administered at a dose of about 1 mg/kg to about 5 mg/kg. In some examples, the ceDNA vector is administered at a dose of about 5 mg/kg to about 20 mg/kg. In some examples, the ceDNA vector is administered at a dose of about 5 mg/kg to about 15 mg/kg. In some examples, the ceDNA vector is administered at a dose of about 5 mg/kg to about 10 mg/kg. In some examples, the ceDNA vector is administered at a dose of about 10 mg/kg to about 20 mg/kg. In some examples, the ceDNA vector is administered at a dose of about 10 mg/kg to about 15 mg/kg. In some examples, the ceDNA vector is administered at a dose of about 15 mg/kg to about 20 mg/kg. In some examples, the ceDNA vector is administered at a dose of about 0.5 mg/kg, 0.75 mg/kg, 1 mg/kg, 1.5 mg/kg, 2 mg/kg, 2.5 mg/kg, 3 mg/kg, 3.5 mg/kg, 4 mg/kg, or 5 mg/kg. In some examples, the ceDNA vector is administered at a dose of about 0.5 mg/kg, 1 mg/kg, 1.5 mg/kg, 2 mg/kg, 2.5 mg/kg, 3 mg/kg, 3.5 mg/kg, 4 mg/kg, or 5 mg/kg.

[0033] In some examples, the administration restores at least about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% of FVIII plasma levels of normal individuals not affected by hemophilia A. In some examples, the administration restores at least about 10% of FVIII plasma levels of normal individuals not affected by hemophilia A. In some examples, the administration restores at least about 15% of FVIII plasma levels of normal individuals not affected by hemophilia A. In some examples, the administration restores at least about 20% of FVIII plasma levels of normal individuals not affected by hemophilia A. In some examples, the administration restores at least about 25% of FVIII plasma levels of normal individuals not affected by hemophilia A. In some examples, the administration restores at least about 30% of FVIII plasma levels of normal individuals not affected by hemophilia A. In some examples, the administration restores at least about 35% of FVIII plasma levels of normal individuals not affected by hemophilia A. In some examples, the administration restores at least about 40% of FVIII plasma levels of normal individuals not affected by hemophilia A. In some examples, the administration restores at least about 45% of FVIII plasma levels of normal individuals not affected by hemophilia A. In some examples, the administration restores at least about 50% of FVIII plasma levels of normal individuals not affected by hemophilia A.

[0034] In some examples of any of the above aspects or examples, the ceDNA vector is administered to a photoreceptor cell, or an RPE cell, or both.

[0035] In some examples of any of the above aspects or examples, the ceDNA vector expresses the FVIII protein in a photoreceptor cell, or an RPE cell, or both.

[0036] In some examples of any of the above aspects or examples, the ceDNA vector is administered by any one or more of subretinal injection, suprachoroidal injection or intravitreal injection.

[0037] In another aspect, the disclosure provides a pharmaceutical composition comprising the ceDNA vector of any one of the aspects or examples herein.

[0038] In another aspect, the disclosure provides a cell containing a ceDNA vector of any of the aspects or examples herein. In some examples, the cell is a photoreceptor cell, or a RPE cell, or both.

[0039] In another aspect, the disclosure provides a composition comprising the ceDNA vector as described herein and a lipid, wherein the lipid is a nanoparticle (LNP). In one example, the disclosure provides a composition comprising a ceDNA vector of any of the aspects or examples herein, and a lipid. In some examples, the lipid is a lipid nanoparticle (LNP). In another example, the disclosure provides a composition comprising a ceDNA vector, wherein the ceDNA vector comprises a nucleic acid sequence at least 85% identical, at least 90% identical, at least 91% identical, at least 92% identical, at least 93% identical, at least 94% identical, at least 95% identical, at least 96% identical, at least 96% identical, at least 97% identical, at least 98% identical or at least 99% identical to, comprises, or consists of SEQ ID NO: 5, and a lipid. In another example, the disclosure provides a composition comprising a ceDNA vector, wherein the ceDNA vector comprises a nucleic acid sequence at least 85% identical, at least 90% identical, at least 91% identical, at least 92% identical, at least 93% identical, at least 94% identical, at least 95% identical, at least 96% identical, at least 96% identical, at least 97% identical, at least 98% identical or at least 99% identical to, comprises, or consists of SEQ ID NO: 42, and a lipid. In some examples, the lipid is an LNP.

[0040] In another aspect, the disclosure provides a kit comprising the ceDNA vector of any of the aspects or examples herein, the pharmaceutical composition of any of the aspects or examples herein, the cell of any of the aspects or examples herein, or the composition of any of the aspects or examples herein.

[0041] In another example, the disclosure provides a capsid-free close-ended DNA (ceDNA) vector comprising at least one nucleic acid sequence between flanking inverted terminal repeats (ITRs), wherein at least one nucleic acid sequence encodes at least one protein, wherein the ceDNA vector comprises a promoter or promoter set operatively linked to the least one nucleic acid sequence that encodes the at least one protein, and wherein the promoter is selected from the group consisting of human a1 antitrypsin (hAAT) promoter, minimal transthyretin promoter (TTRm), hAAT_core_C06, hAAT_core_C07, hAAT_core_08, hAAT_core_C09, hAAT_core_C10, and hAAT_core_truncated. In some examples, the promoter is selected from a nucleic acid sequence having at least 85% identity to any one of SEQ ID NOs: 210-217. In some examples, the promoter set comprises a synthetic liver specific promoter set including enhancers and core promoter, without 5pUTR. In some examples, the promoter set is selected from a nucleic acid sequence having at least 85%, 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%, at least 100% identity to, comprises, or consists of any one of SEQ ID NOs: 184-197, 400, 401, 484, and 617-624.

[0042] In some examples of any of the aspects or examples herein, the ceDNA vector comprises an enhancer. In some examples, the enhancer is selected from the group consisting of: a Serpin enhancer (SerpEnh), the transthyretin (TTRe) gene enhancer (TTRe), the Hepatic Nuclear Factor 1 binding site (HNF1), Hepatic Nuclear Factor 4 binding site (HNF4), Human apolipoprotein E/C-I liver specific enhancer (ApoE_Enh), the enhancer region from Pro-albumin gene (ProEnh), a CpG minimized version of the ApoE_Enh (Human apolipoprotein E/C-I liver specific enhancer) (ApoE_Enh_C03, ApoE_Enh_C04, ApoE_Enh_C09, and ApoE_Enh_C10), and Hepatic nuclear factor enhancer array embedded in GE-856 (Embedded_enhancer_HNF_array). In some examples, the Serpin enhancer comprises a nucleic acid sequence at least 85% identical to SEQ ID NO: 198. In some examples, the enhancer is selected from a nucleic acid sequence having at least 85%, 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%, at least 100% identity to, comprises, or consists of any one of SEQ ID NOs: 198-209, 485 and 557-616.

[0043] In another example, the disclosure provides a method of expressing a protein in a cell comprising contacting the cell with the ceDNA vector of any of the aspects or examples herein. In some examples, the cell is a photoreceptor or a RPE cell. In some examples, the cell is *in vitro* or *in vivo*. In some examples of any of the aspects or examples herein, the at least one nucleic acid sequence is codon optimized for expression in the eukaryotic cell.

[0044] In some examples of any of the aspects or examples herein, the at least one nucleic acid sequence that encodes at least one FVIII protein is selected from a nucleic acid sequence having at least 85% identity to any one of SEQ ID NOs: 556 and 626-633, and wherein the ceDNA vector comprises an enhancer, wherein the enhancer is selected from a nucleic acid sequence having at least 85 % identity to any one of SEQ ID NOs: 557-616.

[0045] In another aspect, the disclosure provides a DNA vector comprising a nucleic acid sequence at least 85% identical to a sequence selected from the group consisting of: SEQ ID NOs: 76, 71-75, 77-183, 556 and 626-633. In one example, the disclosure provides a DNA vector comprising a nucleic acid sequence at least 85% identical to SEQ ID NOs: 71-183, 556 and 626-633. In some examples, the DNA vector comprises an enhancer sequence having at least 95% identity to any one of SEQ ID NOs: 198-209, 485, 557-616. In some examples, the DNA vector comprises a SerpEnh sequence having at least 95% identity to any one of SEQ ID NOs: 198 and 557-616. In some examples, the DNA vector comprises a SerpEnh sequence having at least 95% identity to any one of SEQ ID NOs: 557-616. In some examples, the DNA vector comprises a SerpEnh sequence having at least 95% identity to any one of SEQ ID NOs: 557-568. In some examples, the DNA vector comprises a SerpEnh sequence having at least 95% identity to any one of SEQ ID NOs: 569 and 570.

[0046] In some examples, wherein the DNA vector comprises a SerpEnh sequence having at least 95% identity to any one of SEQ ID NO: 571. In some examples, the DNA vector comprises a SerpEnh sequence having at least 95% identity to any one of SEQ ID NO: 572. In some examples, the DNA vector comprises a SerpEnh sequence having at least 95% identity to any one

of SEQ ID NO: 611. In some examples, the DNA vector comprises a SerpEnh sequence having at least 95% identity to any one of SEQ ID NO: 603.

[0047] In some examples of the aspects and examples herein, the DNA vector comprises a TTRe sequence. In some examples, the TTRe sequence is set forth in SEQ ID NO: 199 or a sequence having at least 95% identity thereof. In some examples, the DNA vector comprises a TTR promoter. In some examples, the TTR promoter is set forth in SEQ ID NO: 211 or a sequence having 95% identity thereof. In some examples, the DNA vector comprises a 5' untranslated region (5' UTR) sequence selected from the group consisting of SEQ ID NO: 411, SEQ ID NO: 412, SEQ ID NO: 413, SEQ ID NO: 414, SEQ ID NO: 415, SEQ ID NO: 416, SEQ ID NO: 417, SEQ ID NO: 418, SEQ ID NO: 419, SEQ ID NO: 420, SEQ ID NO: 421, SEQ ID NO: 422, SEQ ID NO: 423, SEQ ID NO: 424, SEQ ID NO: 425, SEQ ID NO: 426, SEQ ID NO: 427, SEQ ID NO: 428, SEQ ID NO: 429, SEQ ID NO: 430, SEQ ID NO: 431, SEQ ID NO: 432, SEQ ID NO: 433, SEQ ID NO: 434, SEQ ID NO: 435, and SEQ ID NO: 436. In some examples, the DNA vector comprises an intron sequence selected from the group consisting of SEQ ID NO: 235, SEQ ID NO: 236, SEQ ID NO: 237, SEQ ID NO: 238, SEQ ID NO: 239, SEQ ID NO: 240, SEQ ID NO: 241, SEQ ID NO: 242, SEQ ID NO: 243, SEQ ID NO: 245, SEQ ID NO: 246, SEQ ID NO: 247, and SEQ ID NO: 248. In some examples, the DNA vector further comprises an intron sequence having at least 95% identity to SEQ ID NO: 235. In some examples, the DNA vector comprises a 3'UTR sequence. In some examples, the 3'UTR sequence comprises a WPRE element and/or bGH poly A signal sequence or a sequence having at least 95% identity to any one of SEQ ID NOs: 283-291 and 634. In some examples, the DNA vector comprises a microRNA (mir) sequence set forth in SEQ ID NO: 543 or a sequence having at least 95% identity thereof. In some examples, the DNA vector comprises a spacer sequence selected from a sequence having at least 85% identity to any sequence set forth in Table 15 (SEQ ID NOs: 318-332 and 635-641). In some examples, the DNA vector comprises at least one ITR flanking 5' and/or 3' end of the nucleic acid sequence at least 95% identical to SEQ ID NO: 556. In some examples, the at least one ITR flanking 5' and/or 3' is a wild-type AAV ITR(s). In some examples, the DNA vector is a closed-ended DNA (ceDNA). In some examples, the DNA vector is a plasmid. In some examples, the DNA vector comprises a nucleic acid sequence encoding a single chain (SC) FVIII. In some examples, the nucleic acid sequence is set forth in SEQ ID NO: 556 or a sequence having at least 99% identity thereto.

[0048] In another aspect, the disclosure provides a ceDNA vector comprising a nucleic acid sequence selected from the group consisting of: SEQ ID NO: 42, SEQ ID NO: 642, SEQ ID NO: 643, SEQ ID NO: 644, SEQ ID NO: 645 and SEQ ID NO: 646, or a nucleic acid sequence at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a nucleic acid sequence selected from the group consisting of: SEQ ID NO: 42, SEQ ID NO: 642, SEQ ID NO: 643, SEQ ID NO: 644, SEQ ID NO: 645, and SEQ ID NO: 646. In another example, the disclosure provides a ceDNA vector comprising a nucleic acid sequence of SEQ ID NO: 42 or a nucleic acid sequence at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 42.

[0049] In another example, the disclosure provides a ceDNA vector comprising a nucleic acid sequence of SEQ ID NO: 642 or a nucleic acid sequence at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 642.

[0050] In another example, the disclosure provides a ceDNA vector comprising a nucleic acid sequence of SEQ ID NO: 643 or a nucleic acid sequence at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 643.

[0051] In another example, the disclosure provides a ceDNA vector comprising a nucleic acid sequence of SEQ ID NO: 644 or a nucleic acid sequence at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 644.

[0052] In another example, the disclosure provides a ceDNA vector comprising a nucleic acid sequence of SEQ ID NO: 645 or a nucleic acid sequence at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 645.

[0053] In another example, the disclosure provides a ceDNA vector comprising a nucleic acid sequence of SEQ ID NO: 646 or a nucleic acid sequence at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 646.

[0054] These and other aspects of the disclosure are described in further detail below.

DESCRIPTION OF DRAWINGS

[0055] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0056] Embodiments of the present disclosure, briefly summarized above and discussed in greater detail below, can be understood by reference to the illustrative embodiments of the disclosure depicted in the appended drawings. However, the appended drawings illustrate only typical embodiments of the disclosure and are therefore not to be considered limiting of scope, for the disclosure may admit to other equally effective embodiments.

[0057] **FIG. 1A** provides the T-shaped stem-loop structure of a wild-type left ITR of AAV2 (SEQ ID NO: 52) with identification of A-A' arm, B-B' arm, C-C' arm, two Rep binding sites (RBE and RBE') and also shows the terminal resolution site (*TRS*). The RBE contains a series of 4 duplex tetramers that are believed to interact with either Rep 78 or Rep 68. In addition, the RBE' is also believed to interact with Rep complex assembled on the wild-type ITR or mutated ITR in the construct. The D and D' regions contain transcription factor binding sites and other conserved structure. **FIG. 1A** discloses SEQ ID NO: 544. **FIG. 1B** shows proposed Rep-catalyzed nicking and ligating activities in a wild-type left ITR, including the T-shaped stem-loop structure of the wild-type left ITR of AAV2 with identification of A-A' arm, B-B' arm, C-C' arm, two Rep Binding sites (RBE and RBE') and also shows the terminal resolution site (*TRS*), and the D and D' region comprising several transcription factor binding sites and other conserved structure. **FIG. 1B** discloses SEQ ID NO: 545.

CLAIMS

1. A capsid-free close-ended DNA (ceDNA) vector comprising:
at least one nucleic acid sequence between flanking inverted terminal repeats (ITRs),
wherein at least one nucleic acid sequence encodes at least one Factor VIII (FVIII) protein,
wherein the at least one nucleic acid sequence has at least 85% identity to a sequence selected from the group consisting of SEQ ID NOs: 76, 71-75, 77-183, 556 and 626-633.
2. The ceDNA vector of claim 1, wherein the at least one nucleic acid sequence has at least 85% identity to SEQ ID NO: 76.
3. The ceDNA vector of claim 1, wherein the ceDNA vector comprises a promoter or promoter set operatively linked to the least one nucleic acid sequence that encodes at least one FVIII protein.
4. The ceDNA vector of claim 3, wherein the promoter is selected from the group consisting of: a human $\alpha 1$ antitrypsin (hAAT) promoter, a minimal transthyretin promoter (TTRm), an hAAT_core_C06 promoter, an hAAT_core_C07 promoter, an hAAT_core_08 promoter, an hAAT_core_C09 promoter, an hAAT_core_C10 promoter, and an hAAT_core_truncated promoter.
5. The ceDNA vector of claim 3, wherein the promoter comprises a nucleic acid sequence having at least 85% identity to a sequence selected from the group consisting of: SEQ ID NOs: 210-217.
6. The ceDNA vector of claim 3, wherein the promoter set comprises a synthetic liver specific promoter set comprising at least one enhancer and a core promoter, without a 5'UTR.
7. The ceDNA vector of claim 3, wherein the promoter set comprises a nucleic acid sequence having at least 85 % identity to a sequence selected from the group consisting of: SEQ ID NOs: 184-197, 400, 401, and 484.
8. The ceDNA vector of any one of claims 1-7, wherein the ceDNA vector comprises an enhancer.
9. The ceDNA vector of claim 8, wherein the enhancer is selected from the group consisting of: a Serpin enhancer (SerpEnh), a transthyretin (TTRe) gene enhancer (TTRe), a Hepatic Nuclear Factor 1 binding site (HNF1), a Hepatic Nuclear Factor 4 binding site (HNF4), a Human apolipoprotein E/C-I liver specific enhancer (ApoE_Enh), an enhancer region from Pro-albumin gene (ProEnh), a CpG minimized version of the ApoE_Enh (Human apolipoprotein E/C-I liver specific enhancer) (ApoE_Enh_C03, ApoE_Enh_C04, ApoE_Enh_C09, and ApoE_Enh_C10), and a Hepatic nuclear factor enhancer array embedded in GE-856 (Embedded_enhancer_HNF_array).

10. The ceDNA vector of claim 9, wherein the Serpin enhancer comprises a nucleic acid sequence at least 85% identical to SEQ ID NO: 198.
11. The ceDNA vector of claim 8, wherein the enhancer comprises a nucleic acid sequence having at least 85 % identity to a sequence selected from the group consisting of: SEQ ID NOs: 198-209, 485 and 557-616.
12. The ceDNA vector of claim 1, wherein the ceDNA vector comprises a 5' UTR sequence.
13. The ceDNA vector of claim 12, wherein the 5' UTR sequence comprises a sequence having at least 85% identity to a sequence selected from the group consisting of: the sequences set forth in **Table 10**.
14. The ceDNA vector of claim 1, wherein the ceDNA vector comprises an intron sequence.
15. The ceDNA vector of claim 14, wherein the intron sequence comprises a sequence having at least 85% identity to a sequence selected from the group consisting of: the sequences set forth in **Table 11**.
16. The ceDNA vector of claim 1, wherein the ceDNA vector comprises an exon sequence.
17. The ceDNA vector of claim 16, wherein the exon sequence comprises a sequence having at least 85% identity to a sequence selected from the group consisting of: the sequences set forth in **Table 12**.
18. The ceDNA vector of any one of claims 1-17, wherein the ceDNA vector comprises a 3' UTR sequence.
19. The ceDNA vector of claim 18, wherein the 3'UTR sequence comprises a sequence having at least 85% identity to a sequence selected from the group consisting of: the sequences set forth in **Table 13**.
20. The ceDNA vector of claim 1, wherein the ceDNA vector comprises at least one poly A sequence.
21. The ceDNA vector of claim 1, wherein the ceDNA vector comprises one or more DNA nuclear targeting sequences (DTS).
22. The ceDNA vector of claim 21, wherein the DTS comprises a sequence having at least 85% identity to a sequence selected from the group consisting of: the sequences set forth in **Table 14**.
23. The ceDNA vector of claim 1, wherein the ceDNA vector comprises one or more of the following: a Ubiquitous Chromatin-opening Element (UCOE), a Kozak sequence, a spacer sequence, and/or a leader sequence.
24. The ceDNA vector of any one of claims 1-23, wherein the at least one nucleic acid sequence is a cDNA.
25. The ceDNA vector of any one of claims 1-23, wherein at least one flanking ITR comprises a functional terminal resolution site and a Rep binding site.

26. The ceDNA vector of any one of claims 1-25, wherein one or both of the flanking ITRs are derived from a virus selected from a group consisting of: a parvovirus, a dependovirus, and an adeno-associated virus (AAV).
27. The ceDNA vector of any one of claims 1-23, wherein:
the flanking ITRs are symmetric;
the flanking ITRs are substantially symmetrical;
one of the flanking ITRs is a wild-type ITR, or wherein both of the flanking ITRs are wild-type ITRs;
the flanking ITRs are derived from different viral serotypes;
the flanking ITRs are derived from the same viral serotypes; and/or
one or both of the flanking ITRs comprises a sequence selected from the group consisting of: the sequences in **Table 2, Table 4A, Table 4B, and Table 5.**
28. The ceDNA vector of any one of claims 1-27, wherein at least one of the flanking ITRs is altered from a wild-type AAV ITR sequence by a deletion, an addition, and/or a substitution that affects the overall three-dimensional conformation of the ITR.
29. The ceDNA vector of any one of claims 1-28, wherein one or both of the flanking ITRs are derived from an AAV serotype selected from the group consisting of: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, and AAV12.
30. The ceDNA vector of any one of claims 1-29, wherein one or both of the flanking ITRs are synthetic.
31. The ceDNA vector of any one of claims 1-30, wherein one of the flanking ITRs is not a wild-type ITR, or wherein both of the flanking ITRs are not wild-type ITRs.
32. The ceDNA vector of any one of claims 1-31, wherein one or both of the flanking ITRs are modified by a deletion, an insertion, and/or a substitution in at least one of the ITR regions selected from the group consisting of: the A, A', B, B', C, C', D, and D' regions.
33. The ceDNA vector of claim 32, wherein the deletion, insertion, and/or substitution results in the deletion of all or part of a stem-loop structure formed by the A, A', B, B' C, or C' regions.
34. The ceDNA vector of any one of claims 1-37, wherein:
one or both of the flanking ITRs are modified by a deletion, an insertion, and/or a substitution that results in the deletion of all or part of a stem-loop structure formed by the B and B' regions;
one or both of the flanking ITRs are modified by a deletion, an insertion, and/or a substitution that results in the deletion of all or part of a stem-loop structure formed by the C and C' regions;
one or both of the flanking ITRs are modified by a deletion, an insertion, and/or a substitution that results in the deletion of part of a stem-loop structure formed by the B and B' regions and/or all or part of a stem-loop structure formed by the C and C' regions;
one or both of the flanking ITRs comprise a single stem-loop structure in the region that, in a wild-type ITR, would comprise a first stem-loop structure formed by the B and B' regions and a second

stem-loop structure formed by the C and C' regions;
one or both of the flanking ITRs comprise a single stem and two loops in the region that, in a wild-type ITR, would comprise a first stem-loop structure formed by the B and B' regions and a second stem-loop structure formed by the C and C' regions;
one or both of the flanking ITRs comprise a single stem and a single loop in the region that, in a wild-type ITR, would comprise a first stem-loop structure formed by the B and B' regions and a second stem-loop structure formed by the C and C' regions; and/or
both of the flanking ITRs are altered in a manner that results in an overall three-dimensional symmetry when the flanking ITRs are inverted relative to each other.

35. The ceDNA vector of any one of claims 1-34, wherein one or both of the flanking ITRs comprise a sequence selected from the group consisting of: the sequences set forth in **Tables 2, Table 4A, Table 4B, and Table 5**.

36. The ceDNA vector of claim 1, wherein the ceDNA vector comprises a nucleic acid sequence having at least 85% identity with a sequence selected from the group consisting of the sequences set forth in **Table 18**.

37. Use of the ceDNA vector of any one of claims 1-36 in the manufacture of a medicament for expressing an FVIII protein in a cell.

38. The use of claim 37, wherein the cell is a liver cell, a photoreceptor cell, or a retinal pigment epithelial (RPE) cell.

39. The use of claim 37 or 38, wherein the cell is *in vitro* or *in vivo*.

40. The use of any one of claims 37-39, wherein the at least one nucleic acid sequence is codon optimized for expression in the eukaryotic cell.

41. The use of any one of claims 37-40, wherein the at least one nucleic acid sequence has at least 85% identity to a sequence selected from the group consisting of SEQ ID NOs: 76, 71-75, 77-183, 556 and 626-633.

42. Use of the ceDNA vector of any one of claims 1-36 in the manufacture of a medicament for treating a subject with hemophilia A.

43. Use of a nucleic acid sequence having at least 85% identity with a sequence selected from the group consisting of any of the sequences set forth in **Table 18** in the manufacture of a medicament for treating a subject with hemophilia A.

44. The use of claim 42 or claim 43, wherein a level of FVIII in the plasma of the subject is increased in the subject after administration.

45. The use of claim 44, wherein the level of FVIII in the plasma of the subject is increased by at least about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 2-fold, 2.5-fold, 3-fold, 3.5-fold, 4-fold, 5-fold, 10-fold, 15-fold, 20-fold, 25-fold, 30-fold, 40-fold, 50-fold, 60-fold, 70-fold, 80-fold, 90-fold, 100-fold, 200-fold, 300-fold, 400-fold, 500-fold, 600-fold, 700-fold, 800-fold, 900-fold or

1,000-fold after administration.

46. The use of claim 42 or 43, wherein a level of FVIII in the serum of the subject is increased the subject administered the ceDNA vector as compared to a control.

47. The use of claim 46, wherein the increase in the level of FVIII in the serum of the subject is greater than about 40%, 50%, 60%, 70%, 80%, 90%, 100%, 2-fold, 2.5-fold, 3-fold, 3.5-fold, 4-fold, 5-fold, 10-fold, 15-fold, 20-fold, 25-fold, 30-fold, 40-fold, 50-fold, 60-fold, 70-fold, 80-fold, 90-fold, 100-fold, 200-fold, 300-fold, 400-fold, 500-fold, 600-fold, 700-fold, 800-fold, 900-fold or 1,000-fold compared to the control.

48. The use of claim 46 or 47, wherein the control is a level of FVIII in the serum of the subject prior to administration, wherein the control is a level of FVIII in the serum of a subject having hemophilia A who did not receive the administration or wherein the control is a level of FVIII in a subject not having hemophilia A.

49. The use of any one of claims 42-48, wherein the administration restores a plasma level of FVIII in the subject to at least about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% of a FVIII plasma level of a healthy individual not affected by hemophilia A.

50. The use of any one of claims 42-49, wherein the ceDNA vector is to be administered at a dose of about 0.1 mg/kg, 0.2 mg/kg, 0.3 mg/kg, 0.4 mg/kg, 0.5 mg/kg, 0.75 mg/kg, 1 mg/kg, 1.5 mg/kg, 2 mg/kg, 2.5 mg/kg, 3 mg/kg, 3.5 mg/kg, 4 mg/kg, 5 mg/kg, 6 mg/kg, 7 mg/kg, 8 mg/kg, 9 mg/kg, or 10 mg/kg.

51. The use of claim 43, wherein the at least one nucleic acid sequence is a sequence having at least 85% identity to a sequence selected from the group consisting of SEQ ID NOs: 76, 71-75, 77-183, 556 and 626-633.

52. The use of any one of claims 43-51, wherein the ceDNA vector is to be administered to a liver cell, a photoreceptor cell, and/or an RPE cell.

53. The use of any one of claims 43-52, wherein the ceDNA vector expresses the FVIII protein in a liver cell, a photoreceptor cell, or an RPE cell.

54. The use of any one of claims 43-53, wherein the ceDNA vector is to be administered by one or more routes selected from the group consisting of: intravenous injection, intramuscular injection, infusion, subretinal injection, suprachoroidal injection, and intravitreal injection.

55. A pharmaceutical composition comprising the ceDNA vector of any one of claims 1-36.

56. A cell containing the ceDNA vector of any one of claims 1-36.

57. The cell of claim 56, wherein the cell is a liver cell, a photoreceptor cell, and/or an RPE cell.

58. A composition comprising the ceDNA vector of any one of claims 1-36 and a lipid, wherein the lipid is a nanoparticle (LNP).

59. A kit comprising the ceDNA vector of any one of claims 1-36, the pharmaceutical composition of claim 55, the cell of claim 56 or claim 57, or the composition of claim 58.
60. The ceDNA vector of any one of claims 1-35, wherein the at least one nucleic acid sequence that encodes at least one FVIII protein has at least 85% identity to a sequence selected from the group consisting of: SEQ ID NOs: 556 and 626-633, and wherein the ceDNA vector comprises an enhancer, wherein the enhancer has at least 85 % identity to a sequence selected from the group consisting of: SEQ ID NOs: 557-616.
61. A DNA vector comprising a nucleic acid sequence at least 85% identical to a sequence selected from the group consisting of: SEQ ID NOs: 76, 71-75, 77-183, 556 and 626-633.
62. The DNA vector of claim 61, wherein the DNA vector comprises an enhancer sequence having at least 95% identity to a sequence selected from the group consisting of: SEQ ID NOs: 198-209, 485, and 557-616.
63. The DNA vector of claim 61 or 62, wherein the DNA vector further comprises:
a TTR_e sequence;
a TTR promoter;
a 5' untranslated region (5' UTR) sequence selected from the group consisting of: SEQ ID NO: 411, SEQ ID NO: 412, SEQ ID NO: 413, SEQ ID NO: 414, SEQ ID NO: 415, SEQ ID NO: 416 , SEQ ID NO: 417, SEQ ID NO: 418, SEQ ID NO: 419, SEQ ID NO: 420, SEQ ID NO: 421, SEQ ID NO: 422, SEQ ID NO: 423, SEQ ID NO: 424, SEQ ID NO: 425, SEQ ID NO: 426, SEQ ID NO: 427, SEQ ID NO: 428, SEQ ID NO: 429, SEQ ID NO: 430, SEQ ID NO: 431, SEQ ID NO: 432, SEQ ID NO: 433, SEQ ID NO: 434, SEQ ID NO: 435, and SEQ ID NO: 436;
an intron sequence selected from the group consisting of: SEQ ID NO: 235, SEQ ID NO: 236, SEQ ID NO: 237, SEQ ID NO: 238, SEQ ID NO: 239, SEQ ID NO: 240, SEQ ID NO: 241, SEQ ID NO: 242, SEQ ID NO: 243, SEQ ID NO: 245, SEQ ID NO: 246, SEQ ID NO: 247, and SEQ ID NO: 248;
a 3'UTR sequence; and/or
a spacer sequence selected from a sequence having at least 85% identity to any one of the sequences set forth in **Table 15** (SEQ ID NOs:318-332 and 635-641).
64. The DNA vector of claim 63, wherein the 3'UTR sequence comprises a WPRE element and/or a bGH poly A signal sequence or a sequence having at least 95% identity to a sequence selected from the group consisting of: SEQ ID NOs: 283-291 and 634.
65. The DNA vector of claim 63, wherein the DNA vector comprises a microRNA (mir) sequence set forth in SEQ ID NO: 543 or a sequence having at least 95% identity to SEQ ID NO: 543.
66. The DNA vector of claim 61, wherein the DNA vector comprises at least one ITR flanking the 5' and/or the 3' end of the nucleic acid sequence that is at least 95% identical to SEQ

ID NO:556.

67. The DNA vector of claim 66, wherein the at least one ITR flanking the 5' and/or the 3' ends is a wild-type AAV ITR(s).

68. The DNA vector of claim 61, wherein the DNA vector is a closed-ended DNA (ceDNA), or a plasmid.

69. The DNA vector of claim 61, wherein the DNA vector comprises a nucleic acid sequence encoding a single chain (SC) FVIII.

70. The DNA vector of claim 69, wherein the nucleic acid sequence is set forth in SEQ ID NO: 556 or a sequence having at least 99% identity to SEQ ID NO: 556.

71. A ceDNA vector comprising a nucleic acid sequence selected from the group consisting of: SEQ ID NO: 42, SEQ ID NO: 642, SEQ ID NO: 643, SEQ ID NO: 644, SEQ ID NO: 645 and SEQ ID NO: 646, or a nucleic acid sequence at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to a nucleic acid sequence selected from the group consisting of: SEQ ID NO: 42, SEQ ID NO: 642, SEQ ID NO: 643, SEQ ID NO: 644, SEQ ID NO: 645, and SEQ ID NO: 646.