

COMPOSITIONS AND METHODS FOR PRODUCING RECOMBINANT AAV

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority from U.S. Provisional Application 62/806,317, filed February 15, 2019, the content of which is incorporated herein by reference in its entirety.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing that has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on February 13, 2020, is named 025297_WO004_SL.txt and is 47,056 bytes in size.

BACKGROUND OF THE INVENTION

[0003] Adeno-associated virus (AAV) is a small non-enveloped virus belonging to the family *Parvoviridae* and the genus *Dependoparvovirus*. AAV is composed of a single-stranded DNA genome packaged into capsids assembled from three capsid proteins – viral protein (VP) 1, VP2, and VP3 – at an approximate molar ratio of 1:1:10 (Kondratov et al., *Mol Ther.* 25(12):2661-75 (2017)). The capsid proteins are encoded by a single capsid (*cap*) gene and are generated by alternative splicing and differential codon usage (*id.*). VP1 is the largest capsid protein (81.6 kD). VP2 (66.6 kD) is an N-terminal truncated form of VP1. VP3 (59.9 kD) is an N-terminal truncated form of VP2. *See, e.g.,* Cecchini et al., *Hum Gene Ther.* 22:1021-30 (2011).

[0004] Recombinant AAV (rAAV) has been intensively explored as a vector for gene therapy and DNA vaccines in humans. For rAAV production in mammalian cells, a helper virus (e.g., adenovirus, vaccinia, or herpesvirus) is needed. Over the years, several modifications have been made to facilitate the production of rAAV, including (1) identifying and cloning the minimal set of helper proteins (adenovirus E1, E2A, E4, and VA), (2) providing the AAV Rep (Rep78, Rep68, Rep52, and Rep40) and capsid proteins *in trans*, and (3) developing a transgene system that allows packaging of DNA sequences flanked by the AAV Inverted Terminal Repeats (ITRs) (Samulski et al., *J Virol.* 63(9):3822-8 (1989)). When these three components are

introduced into mammalian cells, rAAV containing a transgene of interest can be readily purified. Recombinant AAV so produced has been used in clinical trials (Clement and Grieger, *Mol Ther Methods Clin Dev.* 3:16002 (2016)).

[0005] The need for scaled-up rAAV production for larger clinical trials and commercialization has led to the development of insect cell-based production systems that utilize baculoviral vectors to express the Rep and capsid proteins and to carry the coding sequence for a transgene-containing AAV vector genome, which is packaged into rAAV capsids. Such systems do not require adenovirus helper functions (*see, e.g.,* Urabe et al., *Hum Gene Ther.* 13:1935-43 (2002); Urabe et al., *J Vir.* 80(4):1874-85 (2006); Chen et al., *Mol Ther.* 16(5):924-30 (2008); Smith et al., *Mol Ther.* 17(11):1888-96 (2009); and Mietzsch et al., *Hum Gene Ther Methods* 28(1):15-22 (2017)). While the baculovirus-insect cell system has been successfully utilized for rAAV production at multiple scales, it has been observed that rAAV generated in insect cells has reduced VP1 content (e.g., with a VP1:VP2:VP3 ratio of approximately 1:1:30 to 1:1:60) and consequently reduced potency, as compared to rAAV produced in mammalian cells (*see, e.g.,* Urabe et al., 2002, *supra*; Kohlbrenner et al., *Mol Ther.* 12(6):1217-25 (2005); Urabe et al., 2006, *supra*; Aslanidi et al., *Proc Natl Acad Sci USA* 106(13):5059-64 (2009); Kondratov et al., *supra*; and Mietzsch et al., *supra*).

[0006] Thus, there remains a need for an improved baculovirus-insect cell system for producing rAAV at industrial scales.

SUMMARY OF THE INVENTION

[0007] The present disclosure describes modifications made to the AAV *cap* gene in the baculoviral helper construct that improve the capsid protein ratio, potency, and yield of rAAV produced in a baculovirus-insect cell system.

[0008] In one aspect, the present disclosure provides a nucleic acid construct comprising a nucleotide sequence encoding AAV VP1, VP2, and VP3 proteins, wherein the VP1 protein and the VP2 protein comprise two or more mutations at residues 157, 162, 164, 179, 188, 194, 196, 197, 200, and 201 (numbering according to SEQ ID NO:1) relative to wildtype VP1 and VP2 proteins, respectively. As used herein, “numbering according to SEQ ID NO:1” means amino acid residue positions in SEQ ID NO:1, or the corresponding amino acid residue positions in a different VP1 sequence (e.g., VP1 sequence from a serotype other than AAV6). In some

embodiments, the VP1 protein and the VP2 protein comprise two or more mutations selected from the group consisting of S157A, T162S, Q164A, S179T, L188I, T194A, A196S, A197G, P200S, and T201L (numbering according to SEQ ID NO:1). In further embodiments, the VP1 protein and the VP2 protein comprise all of these ten mutations.

[0009] In some embodiments, the VP1 protein further comprises one or more mutations at residues 67, 81, 84, 85, and 92 relative to wildtype VP1 protein (numbering according to SEQ ID NO:1). In certain embodiments, the VP1 protein comprises one or more mutations selected from the group consisting of A67E, Q81R, K84D, A85S, and R92K. In further embodiments, the VP1 protein comprises all of these five mutations.

[0010] In some embodiments, the VP1 protein and the VP2 protein disclosed herein are identical to the VP1 protein and the VP2 protein, respectively, of AAV6 but for the mutations. In particular embodiments, the VP1 protein and the VP2 protein are derived from AAV6 and comprise mutations S157A, T162S, Q164A, S179T, L188I, T194A, A196S, A197G, P200S, and T201L relative to wildtype AAV6 VP1 and VP2 proteins, respectively.

[0011] In some embodiments of the nucleic acid construct, the nucleotide sequence also comprises an open reading frame coding for assembly-activating protein (AAP), wherein the AAP comprises one or more mutations at residues 8, 10, 12, 17, 21, and 22 (numbering according to SEQ ID NO:10) relative to wildtype AAP protein. As used herein, “numbering according to SEQ ID NO:10” means amino acid residue positions in SEQ ID NO:10, or the corresponding amino acid residue positions in a different AAP sequence (e.g., AAP sequence from a serotype other than AAV6). In certain embodiments, the AAP comprises one or more (e.g., all) mutations selected from the group consisting of P8Q, H10L, L12Q, Q17P, L21Q, and L22V. In particular embodiments, the AAP is identical to wildtype AAP of AAV6 but for the AAP mutations.

[0012] In particular embodiments, the nucleic acid construct of the present disclosure comprises a coding sequence for SEQ ID NO:7, with or without the first amino acid. In further embodiments, the nucleic acid construct comprises nucleotides 18-151 of SEQ ID NO:14. In further embodiments, the nucleic acid construct comprises 10 or more (e.g., 20 or more, 30 or more, 40 or more, 50 or more, 75 or more, 100 or more, 125 or more, or 150 or more) contiguous nucleotides, or the entire nucleotide sequence, of SEQ ID NO:14.

[0013] In one aspect, the present disclosure provides a nucleic acid construct comprising a nucleotide sequence encoding AAV VP1, VP2, and VP3 proteins, wherein the VP1 protein comprises one or more mutations at residues 81, 84, 85, and 92 relative to wildtype VP1 protein (numbering according to SEQ ID NO:1). In some embodiments, the VP1 protein comprises one or more mutations selected from the group consisting of Q81R, K84D, A85S, and R92K. In further embodiments, the VP1 protein comprises all of these four mutations. In particular embodiments, the VP1 protein further comprises a mutation at residue 67, e.g., an A67E mutation. In some embodiments, the VP1 protein is identical to the VP1 protein of AAV6 but for the mutation(s). In certain embodiments, the VP1 protein comprises mutations A67E, Q81R, K84D, A85S, and R92K relative to wildtype AAV6 VP1.

[0014] In one aspect, the present disclosure provides a nucleic acid construct comprising a nucleotide sequence encoding AAP, wherein the AAP comprises one or more mutations at residues 8, 10, 12, 17, 21, and 22 (numbering according to SEQ ID NO:10) relative to wildtype AAP protein. In some embodiments, the AAP comprises one or more (e.g., all) mutations selected from the group consisting of P8Q, H10L, L12Q, Q17P, L21Q, and L22V.

[0015] In some embodiments, the nucleic acid construct of the present disclosure further comprises an AAV *rep* gene.

[0016] In some embodiments, the nucleic acid construct of the present disclosure is a baculoviral vector, wherein the capsid nucleotide sequence is operably linked to a promoter that is active in insect cells.

[0017] The present disclosure also provides an insect cell comprising a presently described nucleic acid construct and a recombinant AAV virion produced in the insect cell.

[0018] The present disclosure also provides a recombinant AAV virion comprising (i) a genome having a transgene of interest flanked by a pair of AAV Inverted Terminal Repeats (ITR), and (ii) a capsid assembled from the VP1, or both VP1 and VP2 proteins, expressed from a presently described nucleic acid construct.

[0019] In yet another aspect, the present disclosure provides a method of producing a recombinant AAV virion, comprising: providing the insect cell described herein, wherein the insect cell also expresses AAV Rep proteins and comprises the coding sequence for an AAV vector comprising a transgene of interest flanked by a pair of AAV ITRs; culturing the insect cell under conditions to allow expression of the VP1, VP2, VP3 proteins, replication of the AAV

vector, and packaging of the AAV vector into a capsid assembled from the expressed VP1, VP2, and VP3 proteins, and recovering the packaged capsid. In some embodiments, the gene(s) encoding the AAV Rep, VP1, VP2 and VP3 proteins reside on one or more nucleic acid constructs. In some embodiments, the gene(s) encoding the AAV Rep proteins reside on one or more nucleic acid constructs that are not the nucleic acid construct(s) expressing the VP1, VP2, and VP3 proteins. In some embodiments, the gene(s) encoding the AAV VP1, VP2 and VP3 proteins reside on one or more nucleic acid constructs that are not the nucleic acid construct(s) expressing the Rep proteins. In some embodiments, the gene(s) encoding the Rep proteins and the gene(s) encoding the VP1, VP2, and VP3 proteins are incorporated into the genome of the insect cell.

[0020] Also provided in the present disclosure are pharmaceutical compositions comprising the present rAAV and a pharmaceutically acceptable carrier.

[0021] Other features, objectives, and advantages of the invention are apparent in the detailed description that follows. It should be understood, however, that the detailed description, while indicating embodiments and aspects of the invention, is given by way of illustration only, not limitation. Various changes and modification within the scope of the invention will become apparent to those skilled in the art from the detailed description.

BRIEF DESCRIPTION OF THE FIGURES

[0022] FIG. 1A shows the alignment of the VP1/VP2 amino acid sequences (SEQ ID NOs:16-21, respectively, in order of appearance) from AAV serotypes 3B, 5, 6, 8, and 9 between amino acid residues 155 and 207 (numbering based on consensus sequence). N-terminal Edman degradation sequencing was performed on the proteolytic cleavage products cored from an SDS-PAGE gel to identify the proteolytic cleavage site in AAV6 VP1/VP2. The cleavage site maps to residues G₁₉₀E₁₉₁. Multiple sequence alignment was generated using the Geneious software package and default settings for ClustalW alignment. The VP3 start site is indicated by the triangle at residue 205.

[0023] FIG. 1B shows the alignment of the VP1/VP2 amino acid sequences (SEQ ID NOs:22-26, 23, and 27-31, respectively, in order of appearance) from AAV serotypes 1, 2, 3B, 4, and 6-11 between amino acid residues 155 and 205 (numbering based on consensus sequence).

Multiple sequence alignment was generated using the Geneious software package and default settings for ClustalW alignment.

[0024] FIG. 1C is a table comparing corresponding amino acid positions from AAV serotypes 1, 4, 6, 7, 8, and 11 between amino acid residues 158-203 as aligned in FIG. 1B. The table indicates amino acid mutations that may prevent proteolytic cleavage in these serotypes. Amino acid residue numbers are based on the consensus sequence shown in Fig. 1B.

[0025] FIGs. 2A-D show that the substitution of a 45-amino acid stretch of an AAV9 VP1/VP2 (SEQ ID NO:4) for the corresponding region in the AAV6 VP1/VP2 sequence (SEQ ID NO:3) abolishes proteolytic cleavage of the resultant chimeric VP1/VP2 proteins ("AAV6/9 VP1/VP2"). FIG. 2A: Coomassie-stained SDS-PAGE gel analysis of purified rAAV6. Asterisk indicates proteolytic cleavage fragment. FIG. 2B: densitometry and capsid ratio analysis of Standard helper and AAV9 Transplant. FIG. 2C: potency comparison of rAAV6 produced in HEK293 cells ("293 AAV") or in Sf9 cells ("Standard Helper" and "AAV9 transplant"). FIG. 2D: alignment of the transplanted AAV9 *cap* nucleotide sequence and the AAV6 *cap* nucleotide sequence that was replaced in AAV9 Transplant; the arrow "A" indicates the AAP start codon. The AAV6 contained a Factor IX ("FIX") transgene, and the virus's potency was assayed by viral transduction of HepG2 cells and subsequent measurement of FIX expression in the cells with ELISA. Potency was calculated relative to rAAV6 produced in HEK293 cells ("293 AAV"). Standard (Std) helper: baculoviral helper vector whose AAV6 *cap* gene does not contain a heterologous sequence from another serotype. AAV9 Transplant: baculoviral helper vector whose AAV6 *cap* gene contains a sequence from the AAV9 *cap* gene, as described above and further below. FIG. 2D discloses SEQ ID NOs:13 and 14, respectively, in order of appearance.

[0026] FIG. 3A is a photograph of a Coomassie-stained SDS-PAGE gel showing that the single point mutation L188I in AAV6 VP1/VP2 did not prevent proteolytic cleavage, as indicated by the protein species pointed to by the arrow. The asterisk denotes an additional cleavage product. Ref: rAAV6 produced in HEK293 cells. L188I: baculoviral AAV6 helper construct encoding AAV6 VP1/VP2 proteins with an L188I mutation (numbering in SEQ ID NO:1).

[0027] FIG. 3B is a bar graph showing the relative potency of rAAV6 produced with baculoviral helper having various AAV *cap* genes. Potency was calculated relative to 293 AAV.

[0028] FIGs. 4A-D illustrate that variants of AAV6/9 VP1/VP2 prevented cleavage, but did not improve potency of the AAV capsid. FIG. 4A: a sequence alignment of Variants 1, 2, 3, and 4 of AAV6/9 VP1/VP2 (numbering according to SEQ ID NO:1). FIG. 4A discloses SEQ ID NOs:32-37, respectively, in order of appearance. FIG. 4B: a photograph of a Coomassie-stained SDS-PAGE gel. *: proteolytic cleavage product. **: additional cleavage product. FIG. 4C: a bar graph showing relative potency of various AAV products. Ref: 293 AAV. Other definitions are the same as above. FIG. 4D: partial alignment of the *cap/AAP* nucleotide sequences and the AAP amino acid sequences (in rectangle) of AAV6, AAV9 Transplant, Variant 1, Variant 2, Variant 3, and Variant 4. The AAP amino acid sequence of AAV9 Transplant has six mutations (P8Q, H10L, L12Q, Q17P, L21Q, and L22V) relative to that of AAP6 in the indicated region, while the AAP amino acid sequences of Variants 1-3 each have only two mutations (L21Q and L22V). Variant 4 has the native AAP6 sequence in the indicated region. FIG. 4D discloses SEQ ID NOs:38-44, 43, 45, 43 and 46-47, respectively, in order of appearance.

[0029] FIGs. 5A and 5B compare the capsid protein ratio and potency of rAAV6 produced with the Standard helper and rAAV6 produced with a baculoviral AAV6 helper construct containing an AAV2 PLA2 (phospholipase A2 domain) transplant (“AAV2 PLA2 transplant” or “AAV6/2 VP1”). The rAAV6 contained a FIX transgene. FIG. 5A: capsid protein ratios of VP1:VP2:VP3 as determined SDS-PAGE and Coomassie Blue densitometry. FIG. 5B: potency comparison of rAAV6 produced in (i) 293 cells or (ii) Sf9 insect cells containing the Standard helper or the AAV6 helper providing an engineered AAV6 VP1 with the AAV2 PLA2 domain. Potency was determined by using FIX cDNA transduction and ELISA for detection of FIX expression, and was calculated relative to 293 AAV.

[0030] FIG. 6 is a graph comparing PLA2 activity *in vitro*. Vg: viral particles.

[0031] FIG. 7A shows the alignment of the assembly-activating protein (AAP) amino acid sequences (SEQ ID NOs:48-52, 8, 53-54, 9 and 55-56, respectively, in order of appearance) from AAV serotypes 1, 2, 3B, 4, and 6-11 in the indicated region. Amino acid residue numbers are based on the consensus sequence. Multiple sequence alignment was generated using the Geneious software package and default settings for ClustalW alignment.

[0032] FIG. 7B shows the amino acid differences in the indicated AAP region (consensus numbering) between (i) AAV1, AAV2, AAV4, AAV6, AAV7, AAV8, AAV10, or AAV11 and

(ii) AAV9 or AAV3B. Amino acid residue numbers are based on the consensus sequence shown in FIG. 7A.

DETAILED DESCRIPTION OF THE INVENTION

[0033] The present disclosure provides improved baculovirus-insect cell systems and related compositions for producing potent rAAV capsids at high yield. In the systems, the *cap* gene in the baculoviral helper construct is mutated in two or more codons such that the VP1 and VP2 proteins encoded by the gene are resistant to proteolytic degradation during the production process. Because the *cap* gene also contains the open reading frame for AAP, these codon changes may also cause mutations in the AAP. The inventors have made the unexpected discovery that the mutations in VP1/VP2 and/or AAP improve the rAAV's infectivity, i.e., potency. Without being bound by theory, the inventors contemplate that the increase in potency may be attributed to the improved integrity of the capsid proteins in insect cells and the improved abilities of the mutated AAP to facilitate capsid assembly.

[0034] Additionally or alternatively, the *cap* gene in the baculoviral helper construct is mutated in one or more codons in the region coding for the VP1 PLA2 domain such that the engineered PLA2 domain acquires higher enzymatic activity. The inventors have made the unexpected discovery that higher PLA2 enzymatic activity may lead to a higher yield of rAAV in the insect cell production system.

[0035] As used herein, when a particular AAV serotype is referred to, it refers to the prototype for the serotype as well as various isolates within the same clade of this prototype. For AAV clade categorization, *see, e.g.*, Gao et al., *J Virol.* (2004) 78(12):6381-8, the disclosure of which is incorporated herein by reference in its entirety.

Removal of Proteolytic Sites in AAV VP1/VP2 Proteins

[0036] In some embodiments, the present improved baculovirus-insect cell systems can be used to produce any AAV serotype that is susceptible to proteolysis in insect cells. Such AAV serotypes may include, for example, AAV1, AAV6, AAV8, or variants thereof, or any pseudotyped or chimeric rAAV whose VP1/VP2 proteins are susceptible to proteolysis in insect cells.

[0037] By “pseudotyped” or “cross-packaged” rAAV is meant a recombinant AAV whose capsid is replaced with the capsid of another AAV serotype, to, for example, alter transduction efficacy or tropism profiles of the virus (e.g., Balaji et al., *J Surg Res.* 184(1):691-8 (2013)). By “chimeric” or “hybrid” rAAV is meant a recombinant AAV whose capsid is assembled from capsid proteins derived from different serotypes and/or whose capsid proteins are chimeric proteins with sequences derived from different serotypes (e.g., serotypes 1 and 2; see, e.g., Hauck et al., *Mol Ther.* 7(3):419-25 (2003)).

[0038] In an improved AAV production system of the present disclosure, two or more point mutations are introduced to AAV VP1/VP2 proteins derived from, e.g., AAV1, AAV4, AAV6, AAV7, AAV8, or AAV11, to remove the sites susceptible to proteolysis in insect cells. The introduced point mutations may be residues identical to those at the corresponding positions in AAV2, AAV3, AAV5, AAV9, or AAV10.

[0039] By “corresponding” amino acid residue or region is meant an amino acid residue or region that aligns with (though not necessarily identical to) the reference residue or region, when the subject sequence and the reference sequence containing the residues or regions are aligned to achieve maximum homology (allowing gaps that are recognized in the art). For example, amino acid residue 189 (L) of AAV8 VP1 corresponds to amino acid residue 188 of AAV6 VP1.

[0040] In some embodiments, the baculoviral helper construct of the present disclosure provides helper functions for the production of rAAV6, and the helper construct includes a modified AAV6 *cap* gene (*cap6*) encoding a mutated VP1 protein. The complete amino acid sequence of an AAV6 VP1 protein is shown below, where the start sites of VP2 (T) and VP3 (M) are boldfaced and underlined:

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1      XAADGYLPDW LEDNLSEGIR EWWDLKPGAP KPKANQQKQD DGRGLVLPGY KYLGPFNGLD
61     KGEFVNAADA AALEHDKAYD QQLKAGDNPY LRYNHADAEF QERLQEDTSF GGNLGRAVFQ
121    AKKRVLEPFG LVEEGAKTAP GKKRPVEQSP QEPDSSSGIG KTGQQPAKKR LNEGQTGDSE
181    SVPDPQPLGE PPATPAAVGP TTMASGGGAP MADNNEGADG VGNASGNWHC DSTWLGD RVI
241    TTSTRTWALP TYNNHLYKQI SSASTGASND NHYFGYSTPW GYFDFNRFHC HFSPRDWQRL
301    INNNWGFPRK RLNFKLENIQ VKEVTNDGV TTIANNLTST VQVFS DSEYQ LPYVLGSAHQ
361    GCLPPFPADV FMIPQYGYLT LNNGSQAVGR SSFYCLEYFP SQMLRTGNF TFSYTFEDVP
421    FHSSYAHSQS LDRLMNPLID QYLYYLNRTQ NQSGSAQNKD LLFSRGSPAG MSVQPKNWLP
481    GPCYRQQRVS KTKTDNNNSN FTWTGASKYN LNGRESIINP GTAMASHKDD KDKFFPMMSGV
541    MIFGKESAGA SNTALDNVMI TDEEEIKATN PVATERFQTV AVNLQSSSTD PATGDVHVMG
601    ALPGMVWQDR DVYLQGPWA KIPHTDGHFH PSPLMGGFGL KHPPPQILIK NTPVPANPPA
661    EFSATKFASF ITQYSTGQVS VEIEWELQKE NSKRWNPEVQ YTSNYAKSAN VDFTVDNNGL
721    YTEPRPIGTR YLTRPL (SEQ ID NO:1)

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In the sequence above, X in position 1 may be M (wildtype; source: GenBank AAB95450.1), T, L, or V. In other embodiments, the VP1 start residue may be another amino acid encoded by a non-canonical start codon such as a suboptimal start codon. *See, e.g., Kearsse et al., Genes Dev.* 31:1717-31 (2017).

[0041] In some embodiments, the above AAV6 VP1/VP2 proteins comprise mutations relative to the wildtype at two or more residues in a region corresponding to residues 138-203 (e.g., residues 151-201 or residues 157-201) of SEQ ID NO:1, where the mutated residues are selected from a group consisting of residues 157, 162, 164, 179, 188, 194, 196, 197, 200, and 201. In some embodiments, the AAV6 VP1/VP2 proteins comprise two or more mutations selected from the group consisting of S157A, T162S, Q164A, S179T, L188I, T194A, A196S, A197G, P200S, and T201L (numbering according to SEQ ID NO:1). For example, an AAV6 VP1/VP2 protein may have the mutations (i) S179T, L188I, T194A, A196S, A197G, P200S, and T201L (“Variant 1”); (ii) S179T, L188I, T194A, A196S, and A197G (“Variant 2”); (iii) T194A, A196S, A197G, P200S, and T201L (“Variant 3”); (iv) S157A, T162S, and Q164A (“Variant 4”); or (v) P200S and T201L. For convenience, only the residue numbers in VP1 are referred to herein. The numbers of the corresponding residues in VP2 can be readily discerned from SEQ ID NO:1 above. For example, residue S157 in VP1 is residue S20 in VP2.

[0042] In particular embodiments, the AAV6 VP1/VP2 proteins comprise the mutation L188I and one or more other mutations in the group. For example, the AAV6 VP1/VP2 proteins may have the mutations L188I, P200S, and T201L.

[0043] In some embodiments, the AAV6 VP1/VP2 proteins comprise all ten mutations of S157A, T162S, Q164A, S179T, L188I, T194A, A196S, A197G, P200S, and T201L, such that their sequence in the region corresponding to residues 157-201 in SEQ ID NO:1 is as follows.

AGIGKSGAQP AKKRLNFGQT GDTESVPDPQ PIGEPPAAPS GVGSL (SEQ ID NO:4)

This sequence is identical to the sequence in the corresponding region in AAV9 VP1/VP2.

[0044] Thus, to generate a modified *cap6* gene in a baculoviral AAV6 helper construct, one can replace the coding sequence for residues 157-201 of VP1 (i.e., residues 20-64 of VP2; SEQ ID NO:3) with the coding sequence for the corresponding amino acid sequence (SEQ ID NO:4) of AAV9, using well known molecular clone techniques; the resultant modified baculoviral AAV6 helper construct is referred to herein as “AAV9 Transplant” and the resultant VP1/VP2 proteins are referred to herein as “AAV6/9 VP1/VP2.” In some embodiments, the

portion of the AAV6 *cap* gene sequence that is replaced comprises the underlined sequence shown below (*see also* FIG. 2D):

AAGAGCCAGACTCCTCCTCGGGCATTGGCAAGACAGGCCAGCAGCCCGCTAAAAAGAGACTCAATTTTGG
TCAGACTGGCGACTCAGAGTCAGTCCCCGACCCACACCTCTCGGAGAACCTCCAGCAACCCCGCTGCT
GTGGGACCTACTACAATGGCTTCAGGCGGTGGCGCACCAATGG (SEQ ID NO:13)

[0045] In certain embodiments, the underlined portion in SEQ ID NO: 13 is replaced by a corresponding *cap* gene sequence from AAV9 to generate an AAV9 Transplant disclosed herein. In particular embodiments, the AAV9 *cap* sequence transplanted to the AAV6 *cap* gene in an AAV9 Transplant comprises the underlined sequence shown below (*see also* FIG. 2D):

AAGAGCCAGACTCCTCCGCGGTATTGGCAAATCGGGTGCACAGCCCGCTAAAAAGAGACTCAATTTTCGG
TCAGACTGGCGACACAGAGTCAGTCCCAGACCTCAACCAATCGGAGAACCTCCCGCAGCCCCCTCAGGT
GTGGGATCTCTTACAATGGCTTCAGGCGGTGGCGCACCAATGG (SEQ ID NO:14)

[0046] In other embodiments, a modified *cap6* gene in the baculoviral AAV6 helper construct can be generated by replacing the coding sequence for residues 157-201 of VP1 in SEQ ID NO:1 (i.e., residues 20-64 of VP2; SEQ ID NO:3) with the coding sequence for the corresponding amino acid sequence from a serotype whose VP1/VP2 proteins are resistant to proteolytic cleavage in insect cells, e.g., AAV2, AAV3, AAV3B, AAV5, or AAV10.

[0047] In other embodiments, the VP1/VP2 proteins of AAV serotypes, such as AAV1, AAV4, AAV7, AAV8, or AAV11, can be mutated such that they contain one or more mutations shown in FIG. 1C. The resultant proteins are expected to be more resistant to proteolysis in insect cells. Unlimiting examples of engineered VP1/VP2 proteins from various AAV serotypes are shown below:

(1) the engineered AAV1 VP1/VP2 proteins may contain one or more of the following mutations: S158T/A, I160T/V, T163A/S/K, Q165K/A, K169R, S180A/T, L189E/I, T195A, A197S/T, A198S/G, V199L, P201T/S, and T202N/L;

(2) the engineered AAV4 VP1/VP2 proteins may contain one or more of the following mutations: T158S/A, I160T/V, K163A/S, K165Q/A, K169R, K171R, V173N, E175G, D176Q/E, E177T, T178G, G179D/E, A180S/T, G181D/E, D182S, G183V, E189L/I, S191Q/E, T192P, S193P, G194A, M196P, S197T, D200G, D201T/S, S202N/L, and E203T;

(3) the engineered AAV6 VP1/VP2 proteins may contain one or more of the following mutations: S158T/A, I160T/V, T163A/S/K, Q165K/A, K169R, S180A/T, L189E/I, T195A, A197S/T, A198S/G, V199L, P201T/S, and T202N/L;

(4) the engineered AAV7 VP1/VP2 proteins may contain one or more of the following mutations: T158S/A, I160T/V, K163A/S, Q165K/A, R169K, S180A/T, L189E/I, S197T, S198G, V199L, and G202N/L;

(5) the engineered AAV8 VP1/VP2 proteins may contain one or more of the following mutations: T158S/A, I160T/V, K163A/S, Q165K/A, R169K, S180A/T, L189E/I, S197T, G198S, V199L, P201T/S, and N202L; and

(6) the engineered AAV11 VP1/VP2 proteins may contain one or more of the following mutations: S158T/A, I160T/V, K163A/S, K165Q/A, R169K, E175G, E176Q, D177T, T178G, G179D/E, A180S/T, G181D/E, D182S, G183V, E189L/I, S191Q/E, D192P, T193P, S194A, M196P, S197T, S200G, D201T/S, I202N/L, and E203T (consensus numbering; *see* FIG. 1C).

[0048] In some embodiments, the VP1/VP2 proteins from an AAV serotype susceptible to proteolysis in insect cells are mutated to contain one or more mutations at the following amino acid residues (consensus numbering in FIG. 1C): 158, 163, 165, 180, 189, 195, 197, 198, 201, and 202. (These residues correspond respectively to amino acid residues 157, 162, 164, 179, 188, 194, 196, 197, 200, and 201 according to the numbering of SEQ ID NO:1 (AAV6) or a corresponding sequence from another serotype aligned to show maximal homology to SEQ ID NO:1.) In further embodiments, the VP1/VP2 proteins may additionally contain one or more mutations at the following amino acid residues (consensus numbering in FIG. 1C): 160, 169, 171-179, 181-183, 191-194, 196, 199, and 203.

[0049] In some embodiments, the VP1/VP2 proteins from an AAV serotype susceptible to proteolysis in insect cells are mutated to contain one or more mutations: S158T/A, I160T/V, K/T163S/A/T, Q165K/A, K169R, K171R, V173N, E175G, D176Q/E, D/E177T, T178G, G179D/E, S180A/T, G181D/E, D182S, G183V, L189E/I, Q/S191E, T/D192P, S/T193P, G/S194A, T/G195A, M196P, A197S/T, A198G/S, V199L, S200G, D/P201S/T, T/S202N/L, and E203T (consensus numbering in FIG. 1C).

[0050] In some embodiments, the engineered VP1/VP2 proteins may contain one or more of the following mutations: S158A, T163S, Q165A, S180T, L189I, T195A, A197S, A198G, P201S, and T202L (consensus number in FIG. 1C). (These residues correspond respectively to

amino acid residues S157A, T162S, Q164A, S179T, L188I, T194A, A196S, A197G, P200S, and T201L according to the numbering of SEQ ID NO:1 (AAV6) or a corresponding sequence from another serotype aligned to show maximal homology to SEQ ID NO:1.)

[0051] The mutations described herein remove sites in the AAV capsid proteins susceptible to proteolytic cleavage in insect cells. Thus, a helper construct containing the modified *cap* gene will give rise to rAAV products of higher purity and uniformity, as well as improved capsid protein.

[0052] Surprisingly, we have found that the point mutations introduced herein to the AAV VP1/VP2 unique region (i.e., region common to VP1 and VP2 but absent in VP3) can also significantly improve the rAAV's potency through a mechanism that does not rely on solely on the prevention of proteolytic cleavage.

Improvement of rAAV Production Yield

[0053] The present disclosure also provides a baculovirus-insect system in which the *cap* gene in the baculoviral helper construct is altered in the VP1 unique region (i.e., region present in VP1 but not in VP2 or VP3) to improve the production yield of the rAAV in insect cells. The VP1 unique region (corresponding to residues 1-137 of SEQ ID NO:1) contains the PLA2 domain, and the mutations in the region may increase the enzymatic activity of the PLA2 domain in the resultant VP1 protein.

[0054] In some embodiments, the baculoviral helper construct of the present disclosure provides helper functions for the production of rAAV6 or rAAV9 and the helper construct includes a modified AAV6 or AAV9 *cap* gene (*cap6* or *cap9*, respectively) with a mutated PLA2 domain. The mutated PLA2 domain may comprise mutations relative to the wildtype at one or more positions in a region corresponding to residues 1-137 (e.g., 52-97 or 67-92) of SEQ ID NO:1, where the positions are selected from a group consisting of residues 67, 81, 84, 85, and 92 (numbering of SEQ ID NO:1). In some embodiments, the AAV6 VP1 protein comprises one or more mutations selected from the group consisting of A67E, Q81R, K84D, A85S, and R92K (numbering according to SEQ ID NO:1). In particular embodiments, the AAV6 VP1 protein comprises all of the five mutations, such that its sequence in the region corresponding to residues 52-97 (SEQ ID NO:5) in SEQ ID NO:1 is as follows.

YLGPFNGLDK GEPVNEADAA ALEHDKAYDR QLDSGDNPYL KYNHAD (SEQ ID NO:6)

This sequence is identical to the sequence in the corresponding region in AAV2 VP1. Thus, to generate a modified *cap6* gene in the baculoviral AAV6 helper construct, one can replace the coding sequence for residues 52-97 or 67-92 of VP1 with the coding sequence for the corresponding amino acid sequence of AAV2, using well known molecular cloning techniques.

[0055] Surprisingly, we have found that the point mutations introduced herein to the AAV VP1 unique region can significantly improve the VP1 PLA2 domain's enzymatic activity and also lead to significant improvement (e.g., two or more fold, three or more fold, four or more fold, or five or more fold) in the yield of the rAAV produced in insect cells.

[0056] In particular embodiments, the engineered *cap* gene encodes an AAV6 VP1 protein containing both the aforementioned mutations in the PLA2 domain and the aforementioned mutations that remove the proteolytic sites. One exemplary modified AAV6 VP1 protein ("AAV6/2/9 VP1") has the following sequence:

```

1      MAADGYLPDW LEDNLSEGIR EWWDLKPGAP KPKANQQKQD DGRGLVLPGY KYLGPFNGLD
61     KGEPVNEADA AALEHDKAYD RQLDSGDNFY LKYNHADA EF QERLQEDTSF GGNLGRAVFQ
121    AKKRVLFPFG LVEEGAKTAP GKKRPVEQSP QEPDSSAGIG KSGAQPAKKR LNFGQTGDTE
181    SVPDPQPIGE PPAAPSGVGS LTMASGGGAP MADNNEGADG VGNASGNWHC DSTWLGD RVI
241    TTSTRTWALP TYNNHLYKQI SSASTGASND NHYFGYSTPW GYFDFNRFHC HFSPRDWQRL
301    INNNWGFRPK RLNFKLFNIQ VKEVTNDGV TTIANNLTST VQVFS DSEYQ LPYVLGSAHQ
361    GCLPPFPADV FMIPQYG YLT LNNGSQAVGR SSFYCLEYFP SQMLRTGN NF TFSYTFEDVP
421    FHSSYAHSQS LDRLMNPLID QYLYYLNRTQ NQSGSAQNKD LLFSRGSPAG MSVQPKNWLP
481    GPCYRQQRVS KTKTDNNNSN FTWTGASKYN LINGRESIINP GTAMASHKDD KDKFFPMSGV
541    MIFGKESAGA SNTALDNVMI TDEEEIKATN PVATERFGTV AVNLQSSSTD PATGDVHVMG
601    ALPGMVWQDR DVYLQGP IWA KIPHTDGHFH PSPLMGGEGL KHPPPQILIK NTPVPANPPA
661    EFSATKFASF ITQYSTGQVS VEIEWELQKE NSKRWNPEVQ YTSNYAKSAN VDFTVDNNGL
721    YTEPRPIGTR YLTRPL (SEQ ID NO:7)

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Other exemplary modified AAV6 VP1 protein has the same sequence as SEQ ID NO:7 except that the start residue in position is not present or is T, L, V, or another amino acid encoded by a non-canonical start codon such as a suboptimal start codon. A baculoviral helper construct encoding one of these modified AAV6 VP1 proteins can be used to produce rAAV6 or a pseudotyped or chimeric rAAV with improved potency and yield in insect cells.

Modifications to AAP

[0057] In another aspect, the present disclosure provides an AAV helper construct encoding an engineered AAP that has improved abilities to stabilize capsid protein and to facilitate capsid assembly. The inventors have discovered that when residues 1-30 of AAV6 AAP ("AAP6") are changed to residues 1-30 AAV9 AAP ("AAP9"), the potency of the rAAV

produced with the engineered helper construct is significantly increased. Residues 1-30 of AAP6 and AAP9 are shown below, where the six amino acid differences in wildtype AAP9 relative to wildtype AAP6 are indicated by boldface and boxes:

AAV6 AAP: MATQSQSPTH NLSENLLQPP LLWDLLQWLQ (SEQ ID NO:8)
 AAV9 AAP: MATQSQS**Q**TL **NQ**SENLL**P**PP **QV**WDLLQWLQ (SEQ ID NO:9)

The complete AAV6 AAP wild-type sequence is shown below:

MATQSQSPTH NLSENLLQPP LLWDLLQWLQ AVAHQWQTIT KAPTEWVMPQ EIGIAIPHW
 ATESSPPAPE HGPCPPITTT STSKSPVLQR GPATTTTTTSA TAPPGGILIS TDSTAISHHV
 TGSDSSTTIG DSGPRDSTSS SSTSRSRRSR RMMASRPSLI TLPARFKSSR TRSTSCRTSS
 ALRTRAASLR SRRTCS (SEQ ID NO:10)

[0058] In some embodiments, the engineered AAP protein comprises one or more mutations at residues 8, 10, 12, 17, 21, and 22 (numbering based on consensus sequence in FIG. 7A). In further embodiments, the engineered AAP protein may comprise one or more of the following amino acid residues: Q8, L10, Q12, P17, Q21, and V22. In some embodiments, the engineered AAP protein may comprise one or more of the following mutations relative to the wildtype AAP sequence: P8Q, H10L, L12Q, Q17P, L21Q, and L22V. Unlimiting examples of engineered, improved AAP proteins are shown below:

- (1) engineered AAP1 (AAP of serotype 1) comprising one or more of the following mutations: P8Q, I9T, H10L, L12Q, L16H, Q17P, L21Q, L22V, and L26I;
- (2) engineered AAP2 (AAP of serotype 2) comprising one or more of the following mutations: E2A, T5S, Y7S, L8Q, P10L, S11N, L12Q, D14E, S15N, H16L, Q17P, L21Q, E24D, I26L, and R27Q;
- (3) engineered AAP4 (AAP of serotype 4) comprising one or more of the following mutations: E2A, Q3T, A4Q, T5S, D6Q, P7S, L12Q, R13S, D14E, Q15N, L16H, P17Q, E18Q, C22V, L23W, M24D, T25L, V26I/L, R27Q, and C28W;
- (4) engineered AAP6 (AAP of serotype 6) comprising one or more of the following mutations: P8Q, H10L, L12Q, L16H, Q17P, L21Q, L22V, and L26I;
- (5) engineered AAP7 (AAP of serotype 7) comprising one or more of the following mutations: P8Q, L12Q, L16H, Q17P, L21Q, L22V, and L26I;
- (6) engineered AAP8 (AAP of serotype 8) comprising one or more of the following mutations: F7S, L12Q, L16H, Q17P, R19P, L21Q, and I26L;
- (7) engineered AAP10 (AAP of serotype 10) comprising one or more of the following mutations: S3T, P8Q, Q12L, L16H, Q17P, A19P, L21Q, and V26I/L; and

(8) engineered AAP11 (AAP of serotype 11) comprising one or more of the following mutations: E2A, P3T, E4Q, T5S, D6Q, P7S, L12Q, R13S, D14E, Q15N, I16L/H, P17Q, A18Q, C22V, L23W, Q24D, T25L, L26I, K27Q, and C28W (*see also* FIG. 7B).

[0059] To generate engineered AAP protein, one can perform point mutations. Because the open reading frame encoding AAP is embedded in the *cap* gene and is translated merely by a frameshift, one may also transplant from another serotype a portion of the *cap* gene containing the coding sequence for an N terminal portion of AAP (e.g., residues 1-30, residues 1-28, residues 2-30, residues 2-28, etc.). In some embodiments, an AAV9 *cap* gene portion coding for an AAV9 VP1/VP2 unique region (i.e., region common to VP1 and VP2 but absent in VP3), for example, a *cap9* sequence comprising nucleotides 18-151 of SEQ ID NO:14 is substituted for the corresponding region of *cap6*, to generate an engineered baculoviral helper construct expressing an engineered AAV6 VP1/VP2 with a portion of AAV9 VP1/VP2 (e.g., so as to remove the proteolytic site(s) as discussed above), as well as an engineered AAP6 whose N-terminal 1-30 amino acid residues are now identical to those of AAP9. In some embodiments, the AAP mutations are generated by the same method as described above for the AAV9 *cap* gene transplant and as further described in Example 1 below.

Production of rAAV in Insect Cells

[0060] Production of rAAV in insect cells can be performed as described previously. *See, e.g.,* Urabe et al., 2002 and 2006, *supra*; Chen et al., *supra*; Smith et al., *supra*; Mietzsch et al., *supra*; WO 2007/046703, WO 2007/148971, WO 2009/104964, WO 2013/036118, and WO 2008/024998, all of which are incorporated herein by reference in their entirety.

[0061] The insect cells in the production methods of the present disclosure comprise a baculoviral helper construct having an expression cassette encoding a modified *cap* gene described herein. The insect cells may be, without limitation, a cultured cell line such as BTI-TN-5B1-4 derived from *Trichoplusia ni* (High Five™, ThermoFisher Scientific, Carlsbad, CA), Sf9 cells or Sf21 cells (both of which are derived from *Spodoptera frugiperda*), Sf9 or TN368 cells with mammalian-type glycan profiles (GlycoBac), or Sf-RVN cells (MilliporeSigma). The insect cells additionally comprise, on the same helper construct or on an additional helper construct, an expression cassette comprising an AAV *rep* gene. The helper construct(s) in the insect cells comprise transcription regulatory elements that direct and regulate the expression of

the Rep proteins and the capsid proteins. These elements include, without limitation: constitutive or inducible promoters that are active in insect cells (e.g., a p5 promoter, a p10 promoter, a p19 promoter, a p40 promoter, a polh promoter, an E1 promoter, and a Δ E1 promoter); Kozak sequences; transcription initiation and termination sites (either modified or unmodified); mRNA splice sites (either modified or unmodified, within or adjacent to the polypeptide coding sequence; and viral, eukaryotic, or prokaryotic RNA elements that control splicing, nuclear export, localization, stabilization, or translation of mRNAs (e.g., Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) (Zufferey et al., *J Virol.* 73(4):2886-92 (1999)), MMLV/MPMV, eukaryotic constitutive transport element (CTE) (Li et al., *Nature* 443(7108):234-7 (2006)), RNA zipcodes (Jambhekar and DeRisi, *RNA* 13(5):625-42 (2007)), and omega or other 5'-UTR RNA elements that increase translational efficiency). The *rep* and/or *cap* gene on the helper construct(s) may be codon-optimized, for example, to modulate expression levels of the polypeptide products, to remove potential undesired transcription/translation initiation sites, to remove cryptic promoter activity, and/or to remove destabilizing elements (e.g., Smith et al., *supra*).

[0062] The rAAV may comprise within its capsid an AAV vector containing a transgene of interest. The transgene may encode a reporter protein for detection using biochemical (luciferase, SEAP) or imaging (GFP, Venus, dTomato) techniques. The transgene may encode a therapeutic protein, including, without limitation, a chimeric antigen receptor (CAR), a C-peptide or insulin, collagen VII, IGF-I, lipoprotein lipase, fibrinogen, prothrombin, Factor V, Factor VIII, Factor IX, Factor XI, Factor XII, Factor XIII, von Willebrand factor, prekallikrein, high molecular weight kininogen (Fitzgerald factor), fibronectin, antithrombin III, heparin cofactor II, protein C, protein S, protein Z, protein Z-related protease inhibitor, plasminogen, alpha 2-antiplasmin, tissue plasminogen activator, urokinase, plasminogen activator inhibitor-1, and plasminogen activator inhibitor-2. The transgene may encode a sequence-specific nuclease (ZFN, TALEN or Cas9) or sequence-specific binding protein (ZFP, TALE or dCas9). The transgene may carry a sequence that can be incorporated into a specific site in the host genome (donor) by homologous recombination to express a therapeutic protein (as described above). The transgene may encode an immunogenic protein for vaccination (e.g., a tumor antigen).

[0063] The AAV vector may comprise transcription regulatory elements (e.g., promoter and enhancer) that can direct and regulate expression of the transgene in human cells. The AAV

vector may also comprise an AAV complete or partial inverted terminal repeat (ITR) on one or both ends of the transgene expression cassette. ITRs are required for packaging and viral integration into the host (human) genome.

Pharmaceutical Compositions of rAAV

[0064] The rAAV capsids produced by the present methods can be formulated with a pharmaceutically acceptable carrier as a pharmaceutical composition. Formulations include, without limitation, suspensions in liquid or emulsified liquids. Pharmaceutically acceptable carriers include, for example, water, saline, dextrose, glycerol, sucrose, or the like, and combinations thereof. In addition, the composition may contain auxiliary substances, such as, wetting or emulsifying agents, pH buffering agents, stabilizing agents, or other reagents that enhance the effectiveness of the rAAV pharmaceutical composition.

[0065] The rAAV pharmaceutical composition may be delivered *in vivo* by administration to the patient, for example, by systemic administration (e.g., intravenous, intraperitoneal, intramuscular, subdermal, intrathecal, or intracranial infusion) or local injections. Alternatively, the rAAV can be delivered to cells *ex vivo*, such as cells explanted from a patient (e.g., lymphocytes, bone marrow aspirates, or tissue biopsy) or allogeneic cells (e.g., universal donor cells such as universal CAR T cells), followed by introduction of the treated cells into the patient, usually after selection for cells that have incorporated the rAAV vector.

[0066] Unless otherwise defined herein, scientific and technical terms used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. Exemplary methods and materials are described below, although methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention. In case of conflict, the present specification, including definitions, will control. Generally, nomenclature used in connection with, and techniques of, cell and tissue culture, molecular biology, virology, immunology, microbiology, genetics, analytical chemistry, synthetic organic chemistry, medicinal and pharmaceutical chemistry, and protein and nucleic acid chemistry and hybridization described herein are those well-known and commonly used in the art. Enzymatic reactions and purification techniques are performed according to manufacturer's specifications, as commonly accomplished in the art or as described herein. Further, unless otherwise required by context, singular terms shall include

pluralities and plural terms shall include the singular. Throughout this specification and embodiments, the words “have” and “comprise,” or variations such as “has,” “having,” “comprises,” or “comprising,” will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers. All publications and other references mentioned herein are incorporated by reference in their entirety. Although a number of documents are cited herein, this citation does not constitute an admission that any of these documents forms part of the common general knowledge in the art.

[0067] In order that this invention may be better understood, the following examples are set forth. These examples are for purposes of illustration only and are not to be construed as limiting the scope of the invention in any manner.

EXAMPLES

[0068] For the following Working Examples, AAV with a transgene comprising a Factor IX (FIX) expression cassette was produced in HEK293 (mammalian) cells using the triple transfection method, or in Sf9 (insect) cells by infecting naïve Sf9 cells with baculovirus-infected insect cells (BIICs) expressing either Rep/Cap protein or providing the AAV genome; the produced AAV was purified using discontinuous cesium chloride density gradients (reviewed in Clement and Grieger, *supra*). Following purification, AAV samples were analyzed by Taqman qPCR using transgene-specific primer probes to quantify viral particle (vg) content. AAV samples ($\sim 2 \times 10^{11}$ vg total) were combined with loading dye and reducing agent, incubated at 95°C for 5 min, loaded on 4-12% NuPAGE™ gels (ThermoFisher Scientific) and electrophoresed for 80 min at 150 V. Gels were stained with SimplyBlue SafeStain (ThermoFisher Scientific) and de-stained according to the manufacturer’s instructions. De-stained gels were scanned using the Li-Cor Odyssey CLx (Li-Cor Biosciences), and bands were quantitated using the Li-Cor Odyssey software. Capsid ratios were calculated relative to VP1 and normalized to the protein molecular weight. To test the potency of AAV, HepG2 cells were transduced with an MOI (multiplicity of infection) of 1×10^6 vg/cell and incubated for 5 days. After 5 days, tissue culture supernatant was collected and FIX protein expression was quantitated using a FIX ELISA kit (Affinity Biologicals, Inc). FIX content in each sample was quantitated based on the standard curve and calculated as relative potency compared to 293 AAV (AAV produced in HEK293 cells).

Example 1: Elimination of Proteolytic Cleavage Sites in rAAV6

[0069] We produced several AAV serotypes in the insect cell system as described above and found that an extra protein fragment was co-purified on cesium chloride density gradient with the expected VP1, VP2, and VP3 proteins for serotypes 6 and 8. This extra protein fragment was seen as an extra band slightly above the VP3 band in Coomassie Blue-stained SDS-PAGE gel, indicating that it was a proteolytic cleavage product of VP1/VP2. This cleavage product was not observed for serotype 3B (Rutledge et al., *J Virol.* 72(1):309-19 (1998)), 5, or 9 produced in insect cells. This cleavage product also was not observed for serotype 6 produced in HEK293 cells. These data suggest that the VP1/VP2 proteins of certain AAV serotypes, e.g., serotypes 6 and 8, are susceptible to proteolytic cleavage in insect cells.

[0070] We performed Edman degradation sequencing on the extra protein fragment from AAV6 and determined that its N-terminal sequence was EPPAT (SEQ ID NO:15), consistent with a proteolytic cleavage site in the sequence PQPLG↓EPPAT (SEQ ID NO:2; “↓” denotes the cleavage point). Sequence alignment of the VP1/VP2 leader sequences of multiple AAV serotypes just before the start of the VP3 protein revealed that this sequence in AAV6 was divergent from serotypes 3B, 5, 8 and 9 (FIGs. 1A and 1B).

[0071] To test whether the proteolytic cleavage could be prevented by altering the sequence around the AAV6 cleavage site, we transferred the nucleotide sequence from AAV9 corresponding to 45 amino acids prior to the VP3 start codon into the AAV6 *cap* (*cap6*) gene in the baculo-helper vector for AAV6 (FIG. 2D). The wildtype AAV6 and AAV9 sequences in this region (residues 157-201 of SEQ ID NO:1) are shown below, where the amino acid changes relative to wildtype AAV6 after the AAV9 sequence transplant are boldfaced and boxed:

AAV6 SGIGKTGQQP AKKRLNFGQT GDSESVDPDPQ PLGEPPATPA AVGPT (SEQ ID NO:3)
 AAV9 **A**GIGK**S**G**A**QP AKKRLNFGQT GD**T**ESVPDPQ P**I**GEPPA**A**P**S** **G**V**G****S**L (SEQ ID NO:4)

[0072] This transfer resulted in ten amino acid changes in total in the resultant AAV6 VP1/VP2 unique region. When rAAV6 was produced using a baculovirus helper construct encoding the engineered VP1/VP2 proteins (“AAV9 Transplant” or “AAV6/9 VP1/VP2”), we found that the proteolytic cleavage was abolished (FIG. 2A), the capsid protein ratio of VP1:VP2:VP3 was improved compared to the standard helper (FIG. 2B), and the potency of

rAAV produced with the AAV9 Transplant helper increased nearly two-fold, compared to the Standard helper (FIG. 2C).

[0073] To dissect the elements in the transferred AAV9 sequence responsible for the improvement, we made a single L188I mutation in the AAV6 VP1/VP2 proteins. While the proteolytic cleavage between residues G₁₈₉ and E₁₉₀ appeared to decrease (FIG. 3A, arrow), a new band above it (FIG. 3A, *) appeared. Thus, the single L188I mutation did not solve the proteolytic problem.

[0074] To determine whether the L188I mutation impacted the potency of the rAAV6 produced from the helper, we produced rAAV containing the FIX cDNA and compared FIX expression levels from cells transduced with rAAV produced in 293 cells (293 AAV) or in Sf9 cells using the standard helper, AAV9 Transplant helper (expressing VP1/VP2 containing the 10 amino acid changes relative to the standard helper), or the L188I mutant helper. Relative potency was calculated with respect to 293 AAV. As shown in FIG. 3B, AAV produced with the AAV9 Transplant helper was significantly more potent than AAV produced with the L188I mutant helper (0.52 vs. 0.31).

[0075] Previous studies suggested that increasing the VP1 content of AAV capsids would lead to increased AAV potency, but most studies attempted to accomplish this by manipulating the start codon or the Kozak sequence near the start codon (Kohlbrenner et al., *supra*); Urabe et al., 2006, *supra*; and Kondratov et al., *supra*). Our work suggests that preventing proteolytic cleavage of VP1 is another viable approach to improving rAAV potency.

[0076] We generated variants (Variants 1-4) of the AAV9 Transplant that contained subsets of the 10 amino acid changes in the AAV9 Transplant (FIG. 4A). As shown in FIG. 4B, while some mutations abolished the proteolytic cleavage (Variant 1 and 3), other mutations either did not abolish proteolytic cleavage (Variant 4) or resulted in an additional proteolytic cleavage (Variant 2). Furthermore, when the potency of these variants was compared to 293 AAV, the standard helper or AAV9 Transplant, no variant achieved equivalent potency to the AAV9 Transplant (FIG. 4C). Thus, although Variants 1 and 3 were not detectably cleaved in insect cells, these two variants did not have improved potency compared to rAAV made with the native AAV6 helper construct.

[0077] We noted that the AAV9 Transplant not only had mutated residues in VP1/VP2 relative to native AAV6 VP1/VP2, the engineered construct also had an altered open reading

frame for AAP, resulting in the following point mutations relative to native AAP6: P8Q, H10L, L12Q, Q17P, L21Q and L22V (FIG. 4D). Variants 1-3 have only two mutations, L21Q and L22V. It appears that one or more of these mutations, e.g., one or more of P8Q, H10L, L12Q, and Q17P, contributed to the observed increase in potency of the rAAV made with the AAV9 Transplant helper construct.

Example 2: Improvement of Recombinant AAV Yield

[0078] Groups working with AAV5 and AAV8 production in the baculovirus-insect cell system reported that rAAV produced in insect cells transduced cells poorly compared to rAAV produced in 293 cells (Kohlbrener et al., *supra*; and Urabe et al., *supra*). These groups transplanted the 134 or 142 amino acid N-terminal portion of the VP1 sequence from AAV2 into the AAV5 or AAV8 VP1 leader sequence and demonstrated that rAAV produced from these chimeric helpers had improved capsid protein ratios and potency with respect to rAAV produced in 293 cells. *Id.* Unfortunately, these studies did not identify the elements in the transplanted sequence that were responsible for the improvement.

[0079] The AAV VP1 leader sequence contains a phospholipase A2-like (PLA2) enzymatic domain, which is required for infection (*see, e.g.,* Zadori et al., *Developmental Cell*, 1:291-302 (2001); and Girod et al., *J of General Virology*, 83:973-8 (2002)). To investigate the role of the PLA2 domain in rAAV's capsid protein ratio, potency, and yield, we transferred an amino acid sequence from AAV2 VP1's PLA2 domain (SEQ ID NO:6) to the baculoviral AAV6 helper, replacing the native AAV6 sequence (SEQ ID NO:5). The wildtype AAV6 and AAV2 sequences in this region (residues 52-97 of SEQ ID NO:1) are shown below, where the five amino acid changes relative to wildtype AAV6 after the AAV2 sequence transplant are boldfaced and boxed:

```
AAV6:  YLGPENGLDK  GEPVNAADAA  ALEHDKAYDQ  QLKAGDNPYL  RYNHAD  (SEQ ID NO:5)
AAV2:  YLGPENGLDK  GEPVNEADAA  ALEHDKAYDQ  QLDSGDNPYL  RYNHAD  (SEQ ID NO:6)
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We observed that there was no significant change in the capsid protein ratios between the Standard helper, which contained the *cap* gene sequence derived entirely from AAV6, and the chimeric baculovirus helper that contained the AAV2 PLA2 transplant ("AAV6/2 VP1") (FIG. 5A). There also was no significant difference in potency between rAAV6 produced with the Standard helper and with the chimeric AAV6/2 helper (FIG. 5B).

[0080] We did, however, observe a significant and reproducible four-fold increase in the yield of rAAV6 produced with the chimeric AAV6/2 helper, as compared to the Standard helper, as shown in Table 1 below, where average yield was calculated based on the yield derived the Standard helper, and N indicates the number of independent production samples. We have found that this result was independent of the sequence being packaged into the rAAV.

Table 1

Helper Construct	Average Yield	N
Standard helper	1.0	7
Chimeric AAV6/2 helper	3.9	7

[0081] Unexpectedly, rAAV6 production using the chimeric AAV6/2 helper resulted in a significant increase in yield compared to the Standard helper.

[0082] We applied the same strategy to a baculovirus AAV9 helper for producing rAAV9 capsid. We replaced the AAV9 VP1 PLA2 domain with that from AAV2 VP1 to generate AAV9/2 VP1. The wildtype AAV9 VP1 sequence (UniProtKB - Q6JC40 (Q6JC40_9VIRU)) is shown below, where the five amino acid residues differing from the corresponding ones in the AAV9/2 VP1 in the PLA2 domain are indicated with boldface and boxes:

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1      MAADGYLPDW LEDNLSEGIR EWWALKPGAP QPKANQQHQD NARGLVLPGY KYLGPGNGLD
61     KGEVNAADA AAEHDKAYD QQLKAGDNPY LKYNHADA EF QERLKEDTSF GGNLGRAVFQ
121    AKKRLLEPLG LVEEAAKTAP GKKRPVEQSP QEPDSSAGIG KSGAQPAKRR LNFGQTGDTE
181    SVPDPQPIGE PPAAPSGVGS LTMASGGGAP VADNNEGADG VGSSSGNWHC DSQWLGDRVI
241    TTSTRTWALP TYNNHLYKQI SNSTSGGSSN DNAYFGYSTP WGYFDENRFH CHFSPRDWQR
301    LINNNWGFRP KRLNFKLFNI QVKEVTDNNG VKTIANNLTS TVQVFTDSDY QLPYVLGSAH
361    EGCLPPFPAD VFMIPQYGYL TLNDGSQAVG RSSFYCLEYF PSQMLRTGNN FQFSYEFENV
421    PFHSSYAHSQ SLDRLMNPLI DQYLYLSKT INGSQQQQT LKFSVAGPSN MAVQGRNYIP
481    GPSYRQQRVS TTVTQNNSE FAWPGASSWA LNGRNSLMNP GPAMASHKEG EDRFFPLSGS
541    LIFGKQGTGR DNVDADKVMI TNEEEIKTTN PVATESYGQV ATNHQSAQAQ AQTGWVQQQ
601    ILPGMVWQDR DVYLQGPIAW KIPHTDGNFH PSPLMGGFGM KHPPPQILIK NTPVPADPPT
661    AFNKDKLNSF ITQYSTGQVS VEIEWELQKE NSKRWNPEIQ YTSNYYKSNN VEFAVNTEGV
721    YSEPRPIGTR YLTRNL (SEQ ID NO:11)

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[0083] The wildtype AAV9 and AAV2 sequences in this region (residues 52-97 of SEQ ID NO:11) are shown below, where the five amino acid changes relative to wildtype AAV9 after the AAV2 sequence transplant are boldfaced and boxed:

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AAV9: YLGPGNGLDK GEPVNAADAA ALEHDKAYDQ QLKAGDNPYL KYNHAD (SEQ ID NO:12)
AAV2: YLGPGNGLDK GEPVNAADAA ALEHDKAYDR QLDSGDNPYL KYNHAD (SEQ ID NO:6)

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[0084] We found that a chimeric helper construct expressing the AAV9/2 VP1 led to a three-fold increase in rAAV9 yield, as compared to the original AAV9 helper containing the native PLA2 domain (Table 2).

Table 2

Helper Construct	Average Yield	N
Standard helper	1.0	3
Chimeric AAV9/2 helper	2.9	3

[0085] We also evaluated the PLA2 enzymatic activity of the AAV6/2 VP1. To do so, we used the ThermoFisher ENZCHEK[®] Phospholipase A2 Assay Kit (E10217). We dissociated the AAV capsids by incubating the viral preparation for 10 min at room temperature in 50 mM NaOH, neutralizing the sample with 100 mM HCl, and then assaying the sample for PLA2 activity according to manufacturer's instructions. Fluorescence emission of the enzyme substrate at 515 nm was recorded. The data show that compared to rAAV6 generated using the standard helper (with and without dissociation), rAAV6 generated with the AAV6/2 helper (helper containing the AAV2 PLA2 domain) had significantly higher PLA2 activity (FIG. 6).

[001] These results suggest that the PLA2 domain possesses additional and previously unknown functions in AAV production, and these functions directly impact the yield in rAAV manufacturing. A helper virus that provides a more enzymatically active PLA2 can help improve rAAV yield in insect cells.

Table 3 List of Sequences

SEQ ID NO	Description
1	AAV6 VP1 amino acid sequence
2	AAV6 proteolytic site
3	AAV6 VP1/VP2 fragment
4	AAV9 VP1/VP2 fragment
5	AAV6 VP1 fragment
6	AAV2 VP1 fragment
7	AAV6/2/9 VP1 amino acid sequence
8	AAP6 residues 1-30
9	AAP9 residues 1-30
10	AAP6 amino acid sequence
11	AAV9 VP1 amino acid sequence
12	AAV9 VP1 fragment
13	Partial AAV6 <i>cap</i> nucleotide sequence
14	Partial AAV9 <i>cap</i> nucleotide sequence
15	AAV6 capsid protein fragment
16	AAV VP1/VP2 partial consensus sequence
17	AAV3B VP1/VP2 fragment
18	AAV5 VP1/VP2 fragment
19	AAV6 VP1/VP2 fragment
20	AAV8 VP1/VP2 fragment
21	AAV9 VP1/VP2 fragment
22	AAV VP1/VP2 partial consensus sequence
23	AAV1 and AAV6 VP1/VP2 fragment
24	AAV2 VP1/VP2 fragment
25	AAV3B VP1/VP2 fragment
26	AAV4 VP1/VP2 fragment
27	AAV7 VP1/VP2 fragment
28	AAV8 VP1/VP2 fragment
29	AAV9 VP1/VP2 fragment
30	AAV10 VP1/VP2 fragment
31	AAV11 VP1/VP2 fragment
32	AAV6 VP1/VP2 fragment
33	AAV6/9 VP1/VP2 fragment (AAV9 transplant)
34	AAV9 transplant variant 1
35	AAV9 transplant variant 2
36	AAV9 transplant variant 3
37	AAV9 transplant variant 4
38	Partial AAV6 <i>cap</i> nucleotide sequence
39	AAV6 AAP fragment
40	Partial AAV9 transplant <i>cap</i> nucleotide sequence
41	AAV9 transplant AAP fragment

42	Partial AAV9 transplant variant 1 <i>cap</i> nucleotide sequence
43	AAV9 transplant variant 1, 2, and 3 AAP fragment
44	Partial AAV9 transplant variant 2 <i>cap</i> nucleotide sequence
45	Partial AAV9 transplant variant 3 <i>cap</i> nucleotide sequence
46	Partial AAV9 transplant variant 4 <i>cap</i> nucleotide sequence
47	AAV9 transplant variant 4 AAP fragment
48	AAV AAP partial consensus sequence
49	AAV1 AAP fragment
50	AAV2 AAP fragment
51	AAV3B AAP fragment
52	AAV4 AAP fragment
53	AAV7 AAP fragment
54	AAV8 AAP fragment
55	AAV10 AAP fragment
56	AAV11 AAP fragment

What is claimed is:

1. A nucleic acid construct comprising a nucleotide sequence encoding adeno-associated virus (AAV) VP1, VP2, and VP3 proteins, wherein the VP1 protein and the VP2 protein comprise two or more mutations at residues 157, 162, 164, 179, 188, 194, 196, 197, 200, and 201 (numbering according to SEQ ID NO:1) relative to wildtype VP1 and VP2 proteins.
2. The nucleic acid construct of claim 1, wherein the VP1 protein and the VP2 protein comprise two or more mutations selected from the first group consisting of S157A, T162S, Q164A, S179T, L188I, T194A, A196S, A197G, P200S, and T201L.
3. The nucleic acid construct of claim 2, wherein the VP1 protein and the VP2 protein comprise all of the mutations in said first group.
4. The nucleic acid construct of any one of the preceding claims, wherein the VP1 protein further comprises one or more mutations at residues 67, 81, 84, 85, and 92 relative to wildtype VP1 protein (numbering according to SEQ ID NO:1).
5. The nucleic acid construct of claim 4, wherein the VP1 protein comprises one or more mutations selected from the second group consisting of A67E, Q81R, K84D, A85S, and R92K.
6. The nucleic acid construct of the claim 5, wherein the VP1 protein comprises all of the mutations in said second group.
7. The nucleic acid construct of any one of the preceding claims, wherein the VP1 protein and the VP2 protein are identical to the VP1 protein and the VP2 protein, respectively, of wildtype AAV6 but for the mutations.
8. The nucleic acid construct of any one of the preceding claims, wherein the nucleotide sequence also comprises an open reading frame coding for assembly-activating protein (AAP),

wherein the AAP comprises one or more mutations at residues 8, 10, 12, 17, 21, and 22 (numbering according to SEQ ID NO:10) relative to wildtype AAP protein.

9. The nucleic acid construct of claim 8, wherein the AAP comprises one or more AAP mutations selected from the third group consisting of P8Q, H10L, L12Q, Q17P, L21Q, and L22V.

10. The nucleic acid construct of claim 9, wherein the AAP is identical to wildtype AAP of AAV6 but for the AAP mutations.

11. The nucleic acid construct of claim 10, wherein the VP1 protein and the VP2 protein are derived from AAV6 and comprise mutations S157A, T162S, Q164A, S179T, L188I, T194A, A196S, A197G, P200S, and T201L relative to AAV6 VP1 and VP2 proteins.

12. The nucleic acid construct of claim 10, wherein the construct comprises a coding sequence for SEQ ID NO:7, with or without the first amino acid.

13. A nucleic acid construct comprising a nucleotide sequence encoding adeno-associated virus (AAV) VP1, VP2, and VP3 proteins, wherein the VP1 protein comprises one or more mutations at residues 81, 84, 85, and 92 relative to wildtype VP1 protein (numbering according to SEQ ID NO:1).

14. The nucleic acid construct of claim 13, wherein the VP1 protein comprises one or more mutations selected from the group consisting of Q81R, K84D, A85S, and R92K.

15. The nucleic acid construct of claim 14, wherein the VP1 protein comprises all of the mutations in said group.

16. The nucleic acid construct of any one of claims 13-15, wherein the VP1 protein further comprises a mutation at residue 67.

17. The nucleic acid construct of claim 16, wherein the VP1 protein comprises an A67E mutation.
18. The nucleic acid construct of any one of claims 13-17, wherein the VP1 protein is identical to the VP1 protein of wildtype AAV6 but for the mutation(s).
19. The nucleic acid construct of claim 18, wherein the VP1 protein comprises mutations A67E, Q81R, K84D, A85S, and R92K relative to AAV6 VP1.
20. A nucleic acid construct comprising a nucleotide sequence encoding assembly-activating protein (AAP), wherein the AAP comprises one or more mutations at residues 8, 10, 12, 17, 21, 22 (numbering according to SEQ ID NO:10) relative to wildtype AAP protein.
21. The nucleic acid construct of claim 20, wherein the AAP comprises one or more mutations selected from the group consisting of P8Q, H10L, L12Q, Q17P, L21Q, and L22V.
22. The nucleic acid construct of any one of the preceding claims, wherein the construct further comprises an AAV *rep* gene.
23. The nucleic acid construct of any one of the preceding claims, wherein the construct is a baculoviral vector and wherein the nucleotide sequence is operably linked to a promoter that is active in insect cells.
24. An insect cell comprising the nucleic acid construct of claim 23.
25. A recombinant AAV virion comprising (i) a genome having a transgene of interest flanked by a pair of AAV Inverted Terminal Repeats (ITR), and (ii) a capsid assembled from the VP1, or both VP1 and VP2 proteins, expressed from the nucleic acid construct of any one of claims 1-23.
26. A recombinant AAV virion produced in the insect cell of claim 24.

27. A method of producing a recombinant AAV virion, comprising:
providing the insect cell of claim 24, wherein the insect cell also expresses AAV Rep proteins and comprises the coding sequence for an AAV vector comprising a transgene of interest flanked by a pair of AAV Inverted Terminal Repeats (ITR),
culturing the insect cell under conditions to allow expression of the VP1, VP2, VP3 proteins, replication of the AAV vector, and packaging of the AAV vector into a capsid assembled from the expressed VP1, VP2, and VP3 proteins, and
recovering the packaged capsid.
28. The method of claim 27, wherein the gene(s) encoding the AAV Rep, VP1, VP2 and VP3 proteins reside on one or more nucleic acid constructs.
29. The method of claim 27 or 28, wherein the gene(s) encoding the AAV Rep proteins reside on one or more nucleic acid constructs that are not the nucleic acid construct(s) expressing the VP1, VP2, and VP3 proteins.
30. The method of any one of claims 27-29, wherein the gene(s) encoding the AAV VP1, VP2 and VP3 proteins reside on one or more nucleic acid constructs that are not the nucleic acid construct(s) expressing the Rep proteins.
31. The method of any one of claims 27-30, wherein the gene(s) encoding the Rep proteins and the gene(s) encoding the VP1, VP2, and VP3 proteins are incorporated into the genome of the insect cell.
32. A recombinant AAV virion produced by the method of any one of claims 27-31.
33. A pharmaceutical composition comprising the recombinant AAV virion of any one of claims 25, 26, and 32 and a pharmaceutically acceptable carrier.