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(54) Title: METHODS FOR PURIFYING ADENO-ASSOCIATED VIRUS VIRIONS

(57) Abstract: The present invention provides methods for purifying adeno-associated virus (AAV) virions, including recombinant AAV virions, generally involving binding of an AAV virion comprising a variant capsid protein comprising a purification peptide to a binding moiety that binds the purification peptide. The present invention further provides nucleic acids comprising nucleotide sequences encoding the variant capsid proteins; as well as recombinant AAV virions comprising the variant capsid proteins.

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METHODS FOR PURIFYING ADENO-ASSOCIATED VIRUS VIRIONS**CROSS-REFERENCE**

- [0001] This application claims the benefit of U.S. Provisional Patent Application No. 60/922,609, filed April 9, 2007, which application is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

- [0002] The U.S. government may have certain rights in this invention, pursuant to grant no. EB003007 awarded by the National Institutes of Health.

BACKGROUND

- [0003] Adeno-associated virus (AAV) is nonpathogenic human parvovirus that requires a helper virus to replicate. The single-stranded 4.6-kb DNA genome of AAV contains two open reading frames (ORFs): *rep*, which encodes a set of four proteins (Rep78, Rep68, Rep52, and Rep40) essential for replication of the viral genome, and *cap*, which encodes the structural proteins (VP1-3). VP1-3 self-assemble into the virus' icosahedral capsid, 25 nm in diameter, into which the genome is inserted. The capsid then escorts the viral genome from the point of entry into tissue to the final viral destination in a target cell nucleus. This high delivery efficiency has been harnessed for gene delivery to and long-term gene expression in a broad array of dividing and nondividing cell types *in vivo*. Natural evolution has generated >100 AAV serotypes, which exhibit a diverse array of gene delivery properties. AAV2 remains the most widely utilized to date, due to its extensive safety record and well-characterized gene delivery properties. In addition, recombinant AAV vectors have been explored in numerous clinical trials.
- [0004] Methods for purifying AAV include precipitation followed by 2-3 rounds of ultracentrifugation through an isopycnic cesium chloride (CsCl) gradient; chromatographic methods, such as affinity chromatography and ion exchange chromatography. For example, heparin and sialic acid affinity resins have been used. However, not all AAV serotypes can bind to existing heparin or sialic acid affinity resins.
- [0005] There is a need in the art for alternative methods for purifying AAV virions, including recombinant AAV virions.

Literature

- [0006] Hu et al. (2003) *Enzyme Microb. Technol.* 33:445-452; Ye et al. (2004) *J. Virol.* 78:9820-9827; Yu and Schaffer (2006) *J. Virol.* 80:3285-3292; Zhang et al. (2002) *J. Virol.* 76:12023-12031; Wu et al. (2000) *J. Virol.* 74:8635-8647; U.S. Patent Application No. 2006/0134740; and Ruan et al. (2004) *Biochem.* 43:14539-14546.

SUMMARY OF THE INVENTION

- [0007] The present invention provides methods for purifying adeno-associated virus (AAV) virions, including recombinant AAV virions, generally involving binding of an AAV virion comprising a variant capsid protein comprising a purification peptide to a binding moiety that binds the purification peptide. The present invention further provides nucleic acids comprising nucleotide sequences encoding the variant capsid proteins; as well as recombinant AAV virions comprising the variant capsid proteins.

BRIEF DESCRIPTION OF THE DRAWINGS

- [0008] Figure 1A depicts a map of an AAV VP3 monomer; and Figure 1B depicts amino acid sequences of wild-type (WT) AAV2, His6 AAV2 clones, and a His6 AAV8 clone.
- [0009] Figures 2A and 2B depict Ni-NTA purification of His6 AAV mutants.
- [0010] Figures 3A-C depict *in vitro* characterization of His6 AAV gene delivery properties.
- [0011] Figures 4A and 4B depict *in vivo* comparison of GFP-expressing vectors with either wild-type (WT) AAV2 or His6 AAV2 capsid.
- [0012] Figures 5A-F depict amino acid sequences of a subtilisin prodomain and variants of same.
- [0013] Figure 6 depicts a subtilisin (mature protein) amino acid sequence.
- [0014] Figure 7 depicts amino acid sequences of variants of a subtilisin polypeptide.
- [0015] Figure 8 depicts the amino acid sequence of an AAV VP2 capsid-subtilisin fusion protein, and the amino acid sequence of an AAV VP2 capsid protein.
- [0016] Figure 9 depicts a nucleotide sequence encoding a subtilisin prodomain.
- [0017] Figure 10 depicts an amino acid sequence of an AAV2 VP1 capsid protein.
- [0018] Figure 11 depicts viral production titer from parent AAV2 and an AAV2 variant ("PalAAV2") comprising a subtilisin domain.
- [0019] Figure 12 depicts infectious elution profile of parent AAV2 and PalAAV2 from a subtilisin affinity column.

DEFINITIONS

- [0020] The terms "affinity peptide," "high affinity peptide," and "metal ion affinity peptide" are used interchangeably herein to refer to a histidine-rich peptide that binds to a metal ion.
- [0021] As used herein, the term "metal ion" refers to any metal ion for which the affinity peptide has affinity and that can be used for purification or immobilization of a fusion protein. Such metal ions include, but are not limited to, Ni^{+2} , Co^{+2} , Fe^{+3} , Al^{+3} , Zn^{+2} and Cu^{+2} . As used herein, the term "hard metal ion" refers to a metal ion that shows a binding preference for oxygen. Hard metal ions include Fe^{3+} , Ca^{2+} , and Al^{3+} . As used herein, the term "soft metal ion" refers to a metal ion that shows a binding preference of sulfur. Soft metal ions include Cu^{+} , Hg^{2+} , and Ag^{+} . As used herein, the term "intermediate metal ion" refers to a metal ion that coordinates nitrogen, oxygen, and sulfur. Intermediate metal ions include Cu^{2+} , Ni^{2+} , Zn^{2+} , and Co^{2+} .
- [0022] A "vector" as used herein refers to a macromolecule or association of macromolecules that comprises or associates with a polynucleotide and which can be used to mediate delivery of the polynucleotide to a cell. Illustrative vectors include, for example, plasmids, viral vectors, liposomes, and other gene delivery vehicles.
- [0023] "AAV" is an abbreviation for adeno-associated virus, and may be used to refer to the virus itself or derivatives thereof. The term covers all subtypes and both naturally occurring and recombinant forms, except where required otherwise. The abbreviation "rAAV" refers to recombinant adeno-associated virus, also referred to as a recombinant AAV vector (or "rAAV vector"). The term "AAV" includes any of a variety of AAV serotypes, including, but not limited to, AAV type 1 (AAV-1), AAV type 2 (AAV-2), AAV type 3 (AAV-3), AAV type 4 (AAV-4), AAV type 5 (AAV-5), AAV type 6 (AAV-6), AAV type 7 (AAV-7), AAV type 8 (AAV-8), avian AAV, bovine AAV, canine AAV, equine AAV, primate AAV, non-primate AAV, and ovine AAV. "Primate AAV" refers to AAV that infect primates, "non-primate AAV" refers to AAV that infect non-primate mammals, "bovine AAV" refers to AAV that infect bovine mammals, etc.
- [0024] An "rAAV vector" as used herein refers to an AAV vector comprising a polynucleotide sequence not of AAV origin (i.e., a polynucleotide heterologous to AAV), typically a sequence of interest for the genetic transformation of a cell. In general, the heterologous polynucleotide is flanked by at least one, and generally by two AAV inverted terminal repeat sequences (ITRs). The term rAAV vector encompasses both rAAV vector particles and rAAV vector plasmids.

- [0025] An "AAV virus" or "AAV viral particle" or "rAAV vector particle" refers to a viral particle composed of at least one AAV capsid protein (typically by all of the capsid proteins of a wild-type AAV) and an encapsidated polynucleotide rAAV vector. If the particle comprises a heterologous polynucleotide (i.e. a polynucleotide other than a wild-type AAV genome such as a transgene to be delivered to a mammalian cell), it is typically referred to as an "rAAV vector particle" or simply an "rAAV vector". Thus, production of rAAV particle necessarily includes production of rAAV vector, as such a vector is contained within an rAAV particle.
- [0026] "Packaging" refers to a series of intracellular events that result in the assembly and encapsidation of an AAV particle.
- [0027] AAV "rep" and "cap" genes refer to polynucleotide sequences encoding replication and encapsidation proteins of adeno-associated virus. AAV rep and cap are referred to herein as AAV "packaging genes."
- [0028] A "helper virus" for AAV refers to a virus that allows AAV (e.g. wild-type AAV) to be replicated and packaged by a mammalian cell. A variety of such helper viruses for AAV are known in the art, including adenoviruses, herpesviruses and poxviruses such as vaccinia. The adenoviruses encompass a number of different subgroups, although Adenovirus type 5 of subgroup C is most commonly used. Numerous adenoviruses of human, non-human mammalian and avian origin are known and available from depositories such as the ATCC. Viruses of the herpes family include, for example, herpes simplex viruses (HSV) and Epstein-Barr viruses (EBV), as well as cytomegaloviruses (CMV) and pseudorabies viruses (PRV); which are also available from depositories such as ATCC.
- [0029] "Helper virus function(s)" refers to function(s) encoded in a helper virus genome which allow AAV replication and packaging (in conjunction with other requirements for replication and packaging described herein). As described herein, "helper virus function" may be provided in a number of ways, including by providing helper virus or providing, for example, polynucleotide sequences encoding the requisite function(s) to a producer cell in trans.
- [0030] An "infectious" virus or viral particle is one that comprises a polynucleotide component which it is capable of delivering into a cell for which the viral species is trophic. The term does not necessarily imply any replication capacity of the virus. Assays for counting infectious viral particles are described elsewhere in this disclosure and in the art. Viral infectivity can be expressed as the P:I ratio, or the ratio of total viral particles to infective viral particles.
- [0031] A "replication-competent" virus (e.g. a replication-competent AAV) refers to a phenotypically wild-type virus that is infectious, and is also capable of being replicated in an infected cell (i.e. in the presence of a helper virus or helper virus functions). In the case of

AAV, replication competence generally requires the presence of functional AAV packaging genes. In general, rAAV vectors as described herein are replication-incompetent in mammalian cells (especially in human cells) by virtue of the lack of one or more AAV packaging genes. Typically, such rAAV vectors lack any AAV packaging gene sequences in order to minimize the possibility that replication competent AAV are generated by recombination between AAV packaging genes and an incoming rAAV vector. In many embodiments, rAAV vector preparations as described herein are those which contain few if any replication competent AAV (rcAAV, also referred to as RCA) (e.g., less than about 1 rcAAV per 10^2 rAAV particles, less than about 1 rcAAV per 10^4 rAAV particles, less than about 1 rcAAV per 10^8 rAAV particles, less than about 1 rcAAV per 10^{12} rAAV particles, or no rcAAV).

[0032] The term "polynucleotide" refers to a polymeric form of nucleotides of any length, including deoxyribonucleotides or ribonucleotides, or analogs thereof. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, and may be interrupted by non-nucleotide components. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The term polynucleotide, as used herein, refers interchangeably to double- and single-stranded molecules. Unless otherwise specified or required, any embodiment of the invention described herein that is a polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double-stranded form.

[0033] A "small interfering" or "short interfering RNA" or siRNA is a RNA duplex of nucleotides that is targeted to a gene interest (a "target gene"). An "RNA duplex" refers to the structure formed by the complementary pairing between two regions of a RNA molecule. siRNA is "targeted" to a gene in that the nucleotide sequence of the duplex portion of the siRNA is complementary to a nucleotide sequence of the targeted gene. In some embodiments, the length of the duplex of siRNAs is less than 30 nucleotides. In some embodiments, the duplex can be 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11 or 10 nucleotides in length. In some embodiments, the length of the duplex is 19-25 nucleotides in length. The RNA duplex portion of the siRNA can be part of a hairpin structure. In addition to the duplex portion, the hairpin structure may contain a loop portion positioned between the two sequences that form the duplex. The loop can vary in length. In some embodiments the loop is 5, 6, 7, 8, 9, 10, 11, 12 or 13 nucleotides in length. The hairpin structure can also contain 3' or 5' overhang portions. In some embodiments, the overhang is a 3' or a 5' overhang 0, 1, 2, 3, 4 or 5 nucleotides in length.

- [0034] "Recombinant," as applied to a polynucleotide means that the polynucleotide is the product of various combinations of cloning, restriction or ligation steps, and other procedures that result in a construct that is distinct from a polynucleotide found in nature. A recombinant virus is a viral particle comprising a recombinant polynucleotide. The terms respectively include replicates of the original polynucleotide construct and progeny of the original virus construct.
- [0035] A "control element" or "control sequence" is a nucleotide sequence involved in an interaction of molecules that contributes to the functional regulation of a polynucleotide, including replication, duplication, transcription, splicing, translation, or degradation of the polynucleotide. The regulation may affect the frequency, speed, or specificity of the process, and may be enhancing or inhibitory in nature. Control elements known in the art include, for example, transcriptional regulatory sequences such as promoters and enhancers. A promoter is a DNA region capable under certain conditions of binding RNA polymerase and initiating transcription of a coding region usually located downstream (in the 3' direction) from the promoter.
- [0036] "Operatively linked" or "operably linked" refers to a juxtaposition of genetic elements, wherein the elements are in a relationship permitting them to operate in the expected manner. For instance, a promoter is operatively linked to a coding region if the promoter helps initiate transcription of the coding sequence. There may be intervening residues between the promoter and coding region so long as this functional relationship is maintained.
- [0037] An "expression vector" is a vector comprising a region which encodes a polypeptide of interest, and is used for effecting the expression of the protein in an intended target cell. An expression vector also comprises control elements operatively linked to the encoding region to facilitate expression of the protein in the target. The combination of control elements and a gene or genes to which they are operably linked for expression is sometimes referred to as an "expression cassette," a large number of which are known and available in the art or can be readily constructed from components that are available in the art.
- [0038] "Heterologous" means derived from a genotypically distinct entity from that of the rest of the entity to which it is being compared. For example, a polynucleotide introduced by genetic engineering techniques into a plasmid or vector derived from a different species is a heterologous polynucleotide. A promoter removed from its native coding sequence and operatively linked to a coding sequence with which it is not naturally found linked is a heterologous promoter. Thus, for example, an rAAV that includes a heterologous nucleic acid encoding a heterologous gene product is an rAAV that includes a nucleic acid not normally

included in a naturally-occurring, wild-type AAV, and the encoded heterologous gene product is a gene product not normally encoded by a naturally-occurring, wild-type AAV.

[0039] The terms "genetic alteration" and "genetic modification" (and grammatical variants thereof), are used interchangeably herein to refer to a process wherein a genetic element (e.g., a polynucleotide) is introduced into a cell other than by mitosis or meiosis. The element may be heterologous to the cell, or it may be an additional copy or improved version of an element already present in the cell. Genetic alteration may be effected, for example, by transfecting a cell with a recombinant plasmid or other polynucleotide through any process known in the art, such as electroporation, calcium phosphate precipitation, or contacting with a polynucleotide-liposome complex. Genetic alteration may also be effected, for example, by transduction or infection with a DNA or RNA virus or viral vector. Generally, the genetic element is introduced into a chromosome or mini-chromosome in the cell; but any alteration that changes the phenotype and/or genotype of the cell and its progeny is included in this term.

[0040] A cell is said to be "stably" altered, transduced, genetically modified, or transformed with a genetic sequence if the sequence is available to perform its function during extended culture of the cell *in vitro*. Generally, such a cell is "heritably" altered (genetically modified) in that a genetic alteration is introduced which is also inheritable by progeny of the altered cell.

[0041] The terms "polypeptide," "peptide," and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The terms also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, lipidation, phosphorylation, or conjugation with a labeling component. Polypeptides such as "CFTR," "p53," "EPO" and the like, when discussed in the context of delivering a gene product to a mammalian subject, and compositions therefor, refer to the respective intact polypeptide, or any fragment or genetically engineered derivative thereof, that retains the desired biochemical function of the intact protein. Similarly, references to CFTR, p53, EPO genes, and other such genes for use in delivery of a gene product to a mammalian subject (which may be referred to as "transgenes" to be delivered to a recipient cell), include polynucleotides encoding the intact polypeptide or any fragment or genetically engineered derivative possessing the desired biochemical function.

[0042] An "isolated" plasmid, nucleic acid, vector, virus, host cell, or other substance refers to a preparation of the substance devoid of at least some of the other components that may also be present where the substance or a similar substance naturally occurs or is initially prepared from. Thus, for example, an isolated substance may be prepared by using a purification technique to enrich it from a source mixture. Enrichment can be measured on an absolute basis,

such as weight per volume of solution, or it can be measured in relation to a second, potentially interfering substance present in the source mixture. Increasing enrichments of the embodiments of this invention are increasingly more isolated. An isolated plasmid, nucleic acid, vector, virus, host cell, or other substance is in some embodiments purified, e.g., from about 80% to about 90% pure, at least about 90% pure, at least about 95% pure, at least about 98% pure, or at least about 99%, or more, pure.

[0043] The terms “individual,” “host,” “subject,” and “patient” are used interchangeably herein, and refer to a mammal, including, but not limited to, human and non-human primates, including simians and humans; mammalian sport animals (e.g., horses); mammalian farm animals (e.g., sheep, goats, etc.); mammalian pets (dogs, cats, etc.); and rodents (e.g., mice, rats, etc.).

[0044] Before the present invention is further described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0045] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0046] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

[0047] It must be noted that as used herein and in the appended claims, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for

example, reference to “a variant capsid protein” includes a plurality of such proteins and reference to “the affinity peptide” includes reference to one or more affinity peptides and equivalents thereof known to those skilled in the art, and so forth. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as “solely,” “only” and the like in connection with the recitation of claim elements, or use of a “negative” limitation.

[0048] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

DETAILED DESCRIPTION

[0049] The present invention provides methods for purifying AAV virions, including recombinant AAV virions, from a composition comprising an AAV virion. The methods generally involve binding of an AAV virion comprising a variant capsid protein to a moiety that binds a purification peptide, where the variant capsid protein comprises the purification peptide. Binding of the AAV virion to the binding moiety allows separation of the AAV virion from undesired molecules that may be present in a preparation comprising the AAV virion. A subject purification method provides for highly-purified, infectious AAV virion particles.

[0050] The present invention further provides nucleic acids comprising nucleotide sequences encoding a variant AAV capsid protein, e.g., a variant AAV capsid protein comprising a purification peptide. The present invention further provides a recombinant AAV virion comprising a variant capsid protein. A recombinant AAV virion can comprise a recombinant AAV vector comprising a heterologous nucleic acid, e.g., a heterologous nucleic acid comprising a nucleotide sequence encoding a gene product of interest, such as a protein that provides a detectable signal, a therapeutic protein, or an interfering nucleic acid.

PURIFICATION METHODS

[0051] The present invention provides methods for purifying AAV virions, including recombinant AAV virions, from a composition comprising an AAV virion. The methods generally involve contacting a composition comprising an AAV virion with a binding moiety, where the AAV virion comprises a variant (“recombinant”) capsid protein comprising a heterologous purification peptide (e.g., an affinity peptide), and where the purification peptide

(e.g., the affinity peptide) binds to the binding moiety, forming a bound AAV virion; and collecting the AAV virion.

[0052] A subject purification method provides for infectious, highly purified AAV virions, e.g., the collected virions are at least 90% pure, at least 95% pure, at least 98% pure, at least 99% pure, or greater than 99% pure. "Infectious" AAV virions are capable of infecting at least one cell type.

[0053] A subject purification method provides for high yield of purified, infectious AAV virions. Thus, the recovery of the AAV virions (e.g., the yield) is at least about 10%, at least about 15%, at least about 20%, at least about 25%, 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, or more.

[0054] In some embodiments, the binding moiety is immobilized on an insoluble support. The nature of the insoluble support can depend, in part, on the nature of the binding moiety. Suitable insoluble supports include, but are not limited to, cross-linked dextrans, polystyrenes, nylon, agarose, cellulose, silica, ceramic, poly(styrenedivinyl)benzene, and polyacrylamides. The insoluble support can be in any of a variety of forms, including, e.g., a bead, a membrane, a filter, and the like. In some embodiments, the insoluble support is in the form of a bead or other particle retained in a column.

[0055] The affinity peptide is a heterologous peptide, e.g., a peptide not normally found in an AAV capsid protein. Suitable affinity peptides include, but are not limited to, metal ion affinity peptides, and protease substrate peptides. Affinity peptides can be synthetic or recombinant, e.g., the affinity peptides have amino acid sequences not normally found in nature.

[0056] Affinity peptides can have a length of from about 5 amino acids to about 150 amino acids, e.g. from about 5 amino acids to about 10 amino acids, from about 10 amino acids to about 15 amino acids, from about 15 amino acids to about 20 amino acids, from about 20 amino acids to about 25 amino acids, from about 25 amino acids to about 30 amino acids, from about 30 amino acids to about 40 amino acids, from about 40 amino acids to about 50 amino acids, from about 50 amino acids to about 60 amino acids, from about 60 amino acids to about 70 amino acids, from about 70 amino acids to about 80 amino acids, from about 80 amino acids to about 90 amino acids, from about 90 amino acids to about 100 amino acids, from about 100 amino acids to about 125 amino acids, or from about 125 amino acids to about 150 amino acids.

[0057] The affinity peptide is present in a fusion protein with an AAV capsid protein. The affinity peptide will in some embodiments be fused at the amino terminus of an AAV capsid protein. In other embodiments, the affinity peptide will be fused at the carboxyl terminus of an AAV capsid protein. In still other embodiments, the affinity peptide will be present at an internal site within the AAV capsid protein. An AAV capsid protein comprising one or more heterologous affinity peptides can be referred to herein as a “variant AAV capsid protein” a “recombinant AAV capsid protein” or “an affinity peptide-containing AAV capsid protein.”

[0058] A variant AAV capsid protein will in some embodiments comprise a single heterologous affinity peptide. In some of these embodiments, an affinity peptide-containing AAV virion comprising the variant capsid protein will comprise a single type of variant capsid protein, e.g., all of the variant capsid proteins in the affinity peptide-containing AAV virion will comprise an affinity peptide of the same amino acid sequence. In other embodiments, an affinity peptide-containing AAV virion comprising a variant capsid protein having a single heterologous affinity peptide will comprise two or more different types of variant capsid proteins. For example, in some embodiments, an affinity peptide-containing AAV virion comprising a variant capsid protein having a single heterologous affinity peptide will comprise a first variant AAV capsid protein having a first single affinity peptide; and at least a second variant AAV capsid protein having a second affinity peptide. For example, in some embodiments, an affinity peptide-containing AAV virion comprising a variant capsid protein having a single heterologous affinity peptide will comprise a first variant AAV capsid protein having a first single affinity peptide that is a metal ion affinity peptide; and at least a second variant AAV capsid protein having a second affinity peptide that is a protease substrate.

[0059] In other embodiments, a variant AAV capsid protein will comprise multiple affinity peptides, e.g., two, three, four, five, or more heterologous affinity peptides, which can be in tandem or dispersed within the AAV capsid protein. In some embodiments, where an affinity peptide-containing AAV capsid protein comprises two or more affinity peptides, each of the two or more affinity peptides has the same amino acid sequence. In other embodiments, where an affinity peptide-containing AAV capsid protein comprises two or more affinity peptides, the two or more affinity peptides can include affinity peptides differing in amino acid sequence from one another. For example, an AAV capsid protein can include a first affinity peptide that is a metal ion affinity peptide; and a second affinity peptide that is a protease substrate.

[0060] An AAV virion that includes an affinity peptide in a capsid protein of the virion can be referred to herein as an “affinity peptide-containing AAV virion”; and the corresponding AAV virion without an affinity peptide in a capsid protein, e.g., an AAV virion that is identical to the

affinity peptide-containing AAV virion but for the affinity peptide, can be referred to herein as the “parent AAV virion.”

[0061] In some embodiments, addition of the affinity peptide to the amino terminus, the carboxyl terminus, or at an internal site of an AAV capsid protein in a virion does not significantly reduce infectivity of the affinity peptide-containing AAV virion for a cell, compared to the infectivity of a parent AAV virion. For example, the presence of the affinity peptide in the affinity peptide-containing AAV virion reduces the infectivity less than about 10-fold, less than about 5-fold, less than 2-fold, less than about 90%, less than 75%, less than about 50%, or less than about 25%, compared to the infectivity of the parent AAV virion for the same cell.

[0062] As noted above, an affinity peptide can be present at an internal site of an AAV capsid protein. For example, an affinity peptide can be present at a site that is one, two, three, four, five, six, seven, eight, nine, 10, or more, amino acids carboxyl-terminal to the amino terminus of an AAV capsid protein; or an affinity peptide can be present at a site that is one, two, three, four, five, six, seven, eight, nine, 10, or more, amino acids amino-terminal to the carboxyl terminus of an AAV capsid protein.

[0063] In some embodiments, each of the capsid proteins present in the affinity peptide-containing AAV virion comprises an affinity peptide. However, it is not necessary that each of the capsid proteins present in the affinity peptide-containing AAV virion comprises an affinity peptide. In some embodiments, the proportion of affinity peptide-containing capsid proteins present in an affinity peptide-containing AAV virion ranges from 10% to about 99%, e.g., from about 10% to about 20%, from about 20% to about 30%, from about 30% to about 40%, from about 40% to about 50%, from about 50% to about 60%, from about 60% to about 70%, from about 70% to about 80%, from about 80% to about 90%, or from about 90% to about 99%, where the remainder of the capsid proteins present in the virion do not include an affinity peptide.

[0064] An affinity peptide-containing AAV capsid protein can be an AAV VP1, VP2, or VP3 polypeptide comprising a heterologous affinity peptide. Where an affinity peptide is present at an internal site in an AAV capsid protein, the affinity peptide can be present at any of a number of locations. As one non-limiting example, an affinity peptide can be inserted in loop III of an AAV capsid protein. As another non-limiting example, an affinity peptide can be inserted in loop IV of an AAV capsid protein (e.g., at a site from amino acid 583-590). As non-limiting examples, the affinity peptide can be inserted between amino acids 585 and 586,

between amino acids 586 and 587, or between amino acids 587 and 588 of an AAV VP2 capsid protein.

[0065] In some embodiments, the purified AAV virion comprises an affinity peptide-containing capsid protein. For example, where the affinity peptide is a metal ion affinity peptide, the metal ion affinity peptide is present in the purified AAV virion. In other embodiments, the affinity peptide is cleaved during the purification process, such that the purified AAV virion does not include the affinity peptide, e.g., the affinity peptide, if present in the purified AAV virion, is present in fewer than about 50%, fewer than about 40%, fewer than about 30%, fewer than about 25%, fewer than about 20%, fewer than about 10%, or fewer than about 5% of the capsid polypeptides present in the AAV virion.

Samples

[0066] A composition (also referred to herein as a “sample”) comprising an AAV virion comprising an affinity peptide-AAV capsid fusion protein (an “affinity peptide-AAV virion”) can be any of a variety of samples comprising an affinity peptide-AAV virion in an unpurified state. In some embodiments, the sample is a lysate of cells that produce an affinity peptide-AAV virion. In some embodiments, a cell lysate is processed, to generate a processed cell lysate, where “processed” can include one or more of centrifugation to remove cellular debris; and the like.

Metal ion affinity peptide-based purification

[0067] In some embodiments, the affinity peptide is a metal ion affinity peptide, and the binding moiety comprises a metal ion. Metal ion affinity peptides, metal ions, insoluble supports comprising a metal ion, and conditions for use of same are described below.

Metal ion affinity peptides

[0068] Metal ion affinity peptides are known in the art, and any metal ion binding peptide, including any known metal ion binding peptide, can be used in a subject recombinant envelope protein. See, e.g., Itakura, et al., *Science* 198:1056-63 (1977); Germino, et al., *Proc. Natl. Acad. Sci. USA* 80:6848-52 (1983); Nilsson et al., *Nucleic Acids Res.* 13:1151-62 (1985); Smith et al., *Gene* 32:321-27 (1984); Dobeli, et al., U.S. Pat. No. 5,284,933; Dobeli, et al., U.S. Pat. No. 5,310,663; U.S. Patent No. 4,569,794; U.S. Patent No. 5,594,115; and U.S. Patent Publication No. 2002/0164718.

[0069] Metal ion affinity peptides include peptides that bind to a metal ion with an affinity of from about 10^3 M^{-1} to about 10^9 M^{-1} , e.g., from about 10^3 M^{-1} to about 10^4 M^{-1} , from about 10^4 M^{-1} to about 10^5 M^{-1} , from about 10^5 M^{-1} to about 10^6 M^{-1} , from about 10^6 M^{-1} to about 10^7 M^{-1} , from about 10^7 M^{-1} to about 10^8 M^{-1} , or from about 10^8 M^{-1} to about 10^9 M^{-1} , or greater than

10^9 M^{-1} . Metal ion affinity peptides can contain from about 30% to about 50%, from about 33% to about 45%, from about 35% to about 43%, or from about 37% to about 40%, histidine residues. For example, a metal ion affinity peptide 18 amino acids in length can contain 6, 7, or 8 histidine residues. Metal ion affinity peptides can be from about 6 to about 30, from about 7 to about 25, from about 8 to about 20, from about 9 to about 18, from about 10 to about 16, or from about 12 to about 14 amino acids in length.

[0070] In some embodiments, a metal ion affinity peptide comprises the amino acid sequence $(\text{His})_n$, where $n = 3-18$, e.g., where $n = 3-6, 6-8, 8-10$, or $10-18$. In some embodiments, $n = 6$.

[0071] In other embodiments, the metal ion affinity peptide comprises an amino acid sequence as set forth in U.S. Patent Publication No. 2002/0164718. In some embodiments, a metal ion affinity peptide comprises a peptide of the formula: $(\text{His}-(\text{X}_1)_n)_m$, wherein $m \geq 3$, wherein X_1 is any amino acid other than His, wherein $n = 1-3$, provided that, in at least one $\text{His}-(\text{X}_1)_n$ unit, $n > 1$.

[0072] In some embodiments, a metal ion affinity peptide comprises a peptide of the formula: $(\text{His}-\text{X}_1-\text{X}_2)_{n1}-(\text{His}-\text{X}_3-\text{X}_4-\text{X}_5)_{n2}-(\text{His}-\text{X}_6)_{n3}$, wherein each of X_1 and X_2 is independently an amino acid with an aliphatic or an amide side chain, each of $\text{X}_3, \text{X}_4, \text{X}_5$ is independently an amino acid with a basic side chain (except His) or an acidic side chain, each X_6 is an amino acid with an aliphatic or an amide side chain, $n1$ and $n2$ are each independently 1-3, and $n3$ is 1-5.

[0073] In some embodiments, each of X_1 and X_2 is independently selected from Leu, Ile, Val, Ala, Gly, Asn, and Gln. In other embodiments, each of X_1 and X_2 is independently selected from Leu, Val, Asn, and Ile. In some embodiments, each of $\text{X}_3, \text{X}_4, \text{X}_5$ is independently selected from Lys, Arg, Asp, and Glu. In some embodiments, each of $\text{X}_3, \text{X}_4, \text{X}_5$ is independently selected from Lys and Glu. In some embodiments, each X_6 is independently selected from Leu, Ile, Val, Ala, Gly, Asn, and Gln. In other embodiments, each X_6 is independently selected from Ala and Asn. For example, the affinity peptide has the amino acid sequence $\text{NH}_2\text{-His-Leu-Ile-His-Asn-Val-His-Lys-Glu-Glu-His-Ala-His-Ala-His-Asn-COOH}$ (SEQ ID NO:17).

[0074] In some embodiments, a metal ion affinity peptide has the formula $(\text{His-Asn})_n$, wherein $n=3$ to 10. In certain embodiments, $n =$ from about 4 to about 10, and preferably from about 5 to about 10. In one particular embodiment, $n=6$.

[0075] In some embodiments, a metal ion affinity peptide has the formula $(\text{His}-\text{X}_1-\text{X}_2)_n$, wherein each of X_1 and X_2 is an amino acid having an acidic side chain, and $n=3$ to 10. In one

embodiment, the affinity peptide comprises the sequence (His-Asp-Asp)₆ (SEQ ID NO:18). In another embodiment, the affinity peptide comprises the sequence (His-Glu-Glu)₆ (SEQ ID NO:19). In a further embodiment, the affinity peptide comprises the sequence (His-Asp-Glu)₆ (SEQ ID NO:20). In a further embodiment, the affinity peptide comprises the sequence (His-Glu-Asp)₆ (SEQ ID NO:21).

Metal ion affinity resins

[0076] When the affinity peptide is a metal ion binding peptide, the binding moiety comprises a metal ion. In some embodiments, the metal ion is immobilized on an insoluble support. Suitable insoluble supports include metal ion chelating resins. Any of a variety of metal ion chelating resins can be used, including commercially available metal ion chelating resins. A metal ion chelating resin can include a carrier matrix, optionally a spacer, and a moiety that comprises a metal ion, e.g., an organic ligand that immobilizes a metal ion. Suitable carrier matrices include, but are not limited to, cross-linked dextrans, polystyrenes, nylon, agarose, and polyacrylamides. Metal chelating ligands include, but are not limited to, carboxymethyl aspartate (CM-Asp); iminodiacetic acid (IDA); tris(carboxymethyl)ethylene diamine (TED); nitrilo triacetic acid (NTA). Several of these are commercially available. A nickel-NTA resin is described in, e.g., U.S. Patent Nos. 4,877,830 and 5,047,513.

[0077] The metal ion chelating resin can be provided in the form of a chromatography column, e.g., wherein the resin is packed in a column. The resin can also comprise a matrix that is a solid support of any shape or configuration. Thus, the term “resin,” as used herein, refers to a resin comprising a matrix in any form, e.g., a bead, a sheet, a well, and the like.

Conditions for binding

[0078] As noted above, in some embodiments, the affinity peptide is a metal ion affinity peptide, and the binding moiety comprises a metal ion, where the metal ion is immobilized on an insoluble support, e.g., a metal ion chelating resin. The conditions under which an AAV virion comprising a capsid protein comprising a metal ion affinity peptide (also referred to herein as a “metal ion affinity AAV virion”) is applied to a metal ion affinity resin will vary according to various parameters, including the metal ion affinity peptide used, the metal ion used, the properties of the undesired components of the sample, etc. Generally, the sample comprising a metal ion affinity AAV virion is applied to the metal ion affinity resin, and the resin is equilibrated with a solution. “Conditions for binding” include a condition of the sample being applied, as well as any equilibration conditions. Those skilled in the art can readily determine appropriate conditions for binding of a metal ion affinity AAV virion in a sample to a metal ion affinity resin.

[0079] The pH conditions suitable for applying a sample comprising a metal ion affinity AAV virion (an AAV virion comprising a capsid protein comprising a metal ion affinity peptide) to a metal ion affinity resin range from about 3.5 to about 11, from about 4.0 to about 10.0, from about 4.5 to about 9.5, from about 5.0 to about 9.0, from about 5.5 to about 8.5, from about 6.0 to about 8.0, or from about 6.5 to about 7.5. Temperature conditions suitable for applying a sample comprising a metal ion affinity peptide-AAV virion to a metal ion affinity resin range from about 15°C to about 40°C, from about 20°C to about 37°C, or from about 22°C to about 25°C. Salt conditions generally range from about 0.01 M to about 1 M, e.g., from about 0.01 M to about 0.05 M, from about 0.05 M to about 0.1 M, from about 0.1 M to about 0.25 M, from about 0.25 M to about 0.5 M, or from about 0.5 M to about 1 M.

[0080] Various additional substances may be included, including, but not limited to, detergents (e.g., sodium dodecyl sulfate, e.g., from about 0.05% to about 2%); non-ionic detergents, e.g., Tween 20™, and the like; chaotropic agents and denaturants, e.g., urea, and guanidinium HCl; buffers, e.g., Tris-based buffers, borate –based buffers, phosphate-based buffers, imidazole, HEPES, PIPES, MOPS, PIPES, TES, and the like.

Washing

[0081] One or more washing steps may be included, to remove undesired components. A washing step may be performed after a metal ion affinity AAV virion is immobilized on a resin. The composition and temperature of a washing solution may vary according to the desired result. The optimal composition and temperature of a washing solution can readily be determined by those skilled in the art, based on known properties of the immobilized metal ion affinity AAV virion. Wash solutions may comprise a buffer, and may further comprise additional components, as necessary, including, but not limited to, a detergent.

Eluting

[0082] The immobilized metal ion affinity AAV virion can be eluted using a pH gradient; addition of a competitor, e.g., an organic acid, phosphates; addition of a displacer such as imidazole; and the like.

Protease substrate-based purification

[0083] In some embodiments, a heterologous affinity peptide is a substrate for a protease. In these embodiments, the binding moiety is a protease that specifically binds to an affinity peptide present in an affinity peptide-containing AAV virion, and that, when activated, cleaves the affinity peptide from the bound AAV virion.

Protease substrates

- [0084] In some embodiments, the affinity peptide is a protease substrate, where the protease substrate is a substrate for subtilisin. Suitable subtilisin substrates include a subtilisin prodomain, or a variant or a fragment of a subtilisin prodomain that is specifically bound by and, under certain conditions, is cleaved by subtilisin from a fusion protein comprising the prodomain.
- [0085] In some embodiments, a subtilisin prodomain has a binding affinity for a subtilisin of from about 10^3 M^{-1} to about 10^9 M^{-1} , e.g., from about 10^3 M^{-1} to about 10^4 M^{-1} , from about 10^4 M^{-1} to about 10^5 M^{-1} , from about 10^5 M^{-1} to about 10^6 M^{-1} , from about 10^6 M^{-1} to about 10^7 M^{-1} , from about 10^7 M^{-1} to about 10^8 M^{-1} , or from about 10^8 M^{-1} to about 10^9 M^{-1} , or greater than 10^9 M^{-1} . In some embodiments, the affinity of the subtilisin prodomain for subtilisin is from about 10^8 M^{-1} to about 10^9 M^{-1} , from about 10^9 M^{-1} to about 10^{10} M^{-1} , from about 10^{10} M^{-1} to about 10^{11} M^{-1} , or from about 10^{12} M^{-1} , or greater than 10^{12} M^{-1} .
- [0086] A subtilisin prodomain can comprise the amino acid sequence depicted in Figure 5A and designated SEQ ID NO:1), or a variant or fragment thereof, where the variant or fragment is specifically bound by and, under certain conditions, is cleaved by subtilisin from a fusion protein comprising the prodomain. In some embodiments, a suitable subtilisin prodomain comprises an amino acid sequence that has at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, or at least about 99% amino acid identity to a contiguous stretch of from about 10 amino acids (aa) to about 15 aa, from about 15 aa to about 20 aa, from about 20 aa to about 25 aa, from about 25 aa to about 30 aa, from about 30 aa to about 40 aa, from about 40 aa to about 50 aa, from about 50 aa to about 60 aa, from about 60 aa to about 70 aa, or from about 70 aa to about 77 aa, of the amino acid sequence depicted in Figure 5A (SEQ ID NO:1). Suitable subtilisin prodomains are described in, e.g., U.S. Patent Publication No. 2006/0134740; and Ruan et al. (2004) *Biochemistry* 43:14539.
- [0087] In some embodiments, a suitable subtilisin prodomain has a length of from about 9 amino acids to about 77 amino acids, e.g., from about 9 aa to about 15 aa, from about 15 aa to about 20 aa, from about 20 aa to about 25 aa, from about 25 aa to about 30 aa, from about 30 aa to about 40 aa, from about 40 aa to about 50 aa, from about 50 aa to about 60 aa, from about 60 aa to about 70 aa, or from about 70 aa to about 80 aa.
- [0088] In some embodiments, a suitable subtilisin prodomain comprises the amino acid sequence EEDKL(F/Y)(Q/K)(S/A)(M/L/Y) (SEQ ID NO:22). In some embodiments, a suitable subtilisin prodomain comprises the amino acid sequence EEDKL(F/Y)(Q/K)(S/A)(M/L/Y) (SEQ ID NO:22) at or near the carboxyl terminus of the

prodomain, and has a length of 9 aa, 10 aa, 10-20 aa, 20-30 aa, 30-40 aa, 40-50 aa, 50-60 aa, 60-70 aa, or 70-80 aa, or longer than 80 aa.

[0089] In some embodiments, a suitable subtilisin prodomain comprises, compared to the amino acid sequence set forth in SEQ ID NO:1, from one to about 20, or from one to ten, amino acid substitutions and/or deletions and/or insertions. In some embodiments, a suitable subtilisin prodomain comprises, compared to the amino acid sequence set forth in SEQ ID NO:1, one or more of the following amino acid sequence changes: 1) a substitution of QTMSTM (SEQ ID NO:23; amino acids 16-21) with SGIK (SEQ ID NO:24); 2) an A23C substitution; 3) a K27Q substitution; 4) a V37L substitution; 5) a Q40C substitution; 6) an H72K substitution; 7) an H75K substitution; 8) an A74F substitution; 9) an H75K substitution; and 10) a Y77M substitution. In some embodiments, a suitable subtilisin prodomain comprises, compared to the amino acid sequence set forth in SEQ ID NO:1, the following changes: 1) a substitution of QTMSTM (SEQ ID NO:23; amino acids 16-21) with SGIK (SEQ ID NO:24); 2) an A23C substitution; 3) a K27Q substitution; 4) a V37L substitution; and 5) a Q40C substitution. In some embodiments, a suitable subtilisin prodomain comprises, compared to the amino acid sequence set forth in SEQ ID NO:1, the following changes: 1) a substitution of QTMSTM (SEQ ID NO:23; amino acids 16-21) with SGIK (SEQ ID NO:24); 2) an A23C substitution; 3) a K27Q substitution; 4) a V37L substitution; 5) a Q40C substitution; and 6) substitutions of one or more of amino acids 74-77 (e.g., substitution of A74-H75-A76-Y77 with one of: i) FKAM (SEQ ID NO:25); ii) FKAF (SEQ ID NO:26); iii) FKAY (SEQ ID NO:27); or iv) FKAL (SEQ ID NO:28). In some embodiments, a suitable subtilisin prodomain comprises, compared to the amino acid sequence set forth in SEQ ID NO:1, the following changes: 1) a substitution of QTMSTM (SEQ ID NO:23; amino acids 16-21) with SGIK (SEQ ID NO:24); 2) an A23C substitution; 3) a K27Q substitution; 4) a V37L substitution; 5) a Q40C substitution; and 6) substitution of A74-H75-A76-Y77 with FKAL (SEQ ID NO:28). In some embodiments, a suitable subtilisin prodomain comprises, compared to the amino acid sequence set forth in SEQ ID NO:1, one or more of the amino acid substitutions depicted in bold in Figures 5B-5F. In some embodiments, a suitable subtilisin prodomain comprises an amino acid sequence as set forth in one of Figures 5B-5F.

[0090] In some embodiments, the subtilisin prodomain is fused to the amino terminus of an AAV capsid protein. For example, in some embodiments, the subtilisin prodomain is fused to the amino terminus of VP2. A non-limiting example of an AAV capsid protein comprising a subtilisin prodomain is shown in Figure 8 ("AAV2 prodomain-VP2"; SEQ ID NO:13).

[0091] In some embodiments, an affinity peptide-containing AAV virion comprises a VP2 capsid fusion protein with a subtilisin prodomain at the amino terminus of the VP2 polypeptide. In some of these embodiments, the AAV is AAV2. In other embodiments, the AAV is AAV8. In other embodiments, the AAV is a serotype other than AAV2 or AAV8.

Proteases

[0092] Where the affinity peptide is a protease substrate, the binding moiety is a protease that specifically binds to the affinity peptide and, under certain conditions, cleaves the affinity peptide from an affinity peptide-containing AAV virion or the affinity peptide-containing AAV capsid protein. A suitable protease catalyzes cleavage of the affinity peptide from an affinity peptide-containing AAV capsid protein comprising the affinity peptide; and does not cleave internally within the capsid protein itself. For example, a suitable protease specifically cleaves only at the junction between the affinity peptide and the capsid protein.

[0093] In some embodiments, a suitable protease binds specifically to an affinity peptide present in an affinity peptide-AAV capsid fusion polypeptide, but does not substantially cleave the affinity peptide from the affinity peptide-AAV capsid fusion polypeptide except under certain conditions, e.g., "activation" conditions. For example, addition of an activator can trigger the cleavage of the affinity peptide from the affinity peptide-AAV capsid fusion polypeptide. In the absence of the activator, cleavage of the affinity peptide from the affinity peptide-AAV capsid fusion polypeptide occurs, if at all, at a very low rate (e.g., $k_{cat}/K_m \sim 1 \text{ M}^{-1} \text{ s}^{-1}$ in 0.1 M KPO₄ at pH 7.2 and 25°C).

[0094] Where the affinity peptide is a subtilisin prodomain, the binding moiety is a subtilisin polypeptide. Suitable subtilisin polypeptides include polypeptides having at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98, or at least about 99% amino acid identity to a contiguous stretch of from about 50 amino acids to about 100 amino acids, from about 100 amino acids to about 150 amino acids, from about 150 amino acids to about 200 amino acids, or from about 200 amino acids to about 275 amino acids, of the amino acid sequence depicted in Figure 6 (SEQ ID NO:7). Suitable subtilisin polypeptides are described in, e.g., U.S. Patent Publication No. 2006/0134740; and Ruan et al. (2004) *Biochemistry* 43:14539.

[0095] In some embodiments, a suitable subtilisin polypeptide is a variant subtilisin and comprises, compared to the amino acid sequence set forth in Figure 6 (SEQ ID NO:7), one or more of the following changes: 1) a Q2K substitution; 2) an S3C substitution; 3) a P5S substitution; 4) an S9A substitution; 5) an I31L substitution; 6) a K43N substitution; 7) an M50F substitution; 8) an A73L substitution; 9) deletion of amino acids 75-83; 10) an E156S

substitution; 11) a G166S substitution; 12) a G169A substitution; 13) an S188P substitution; 14) a Q206C substitution; 15) an N212G substitution; 16) a Y217L substitution; 17) an N218S substitution; 18) a T254A substitution; 19) a S271E substitution; 20) a Y104A substitution; 21) a G128S substitution; 22) a D32A substitution; 23) a D32A substitution; and 24) a D32V substitution.

[0096] In some embodiments, a suitable subtilisin polypeptide is a variant subtilisin and comprises, compared to the amino acid sequence set forth in Figure 6 (SEQ ID NO:7), all of the following changes: 1) a Q2K substitution; 2) an S3C substitution; 3) a P5S substitution; 4) an S9A substitution; 5) an I31L substitution; 6) a K43N substitution; 7) an M50F substitution; 8) an A73L substitution; 9) deletion of amino acids 75-83; 10) an E156S substitution; 11) a G166S substitution; 12) a G169A substitution; 13) an S188P substitution; 14) a Q206C substitution; 15) an N212G substitution; 16) a Y217L substitution; 17) an N218S substitution; 18) a T254A substitution; and 19) a S271E substitution. The amino acid sequence such a variant is depicted in Figure 7A (SEQ ID NO:8), where the underlined residues 75-83 are deleted.

[0097] In some embodiments, a suitable subtilisin polypeptide is a variant subtilisin and comprises, compared to the amino acid sequence set forth in Figure 6 (SEQ ID NO:7), all of the following changes: 1) a Q2K substitution; 2) an S3C substitution; 3) a P5S substitution; 4) an S9A substitution; 5) an I31L substitution; 6) a K43N substitution; 7) an M50F substitution; 8) an A73L substitution; 9) deletion of amino acids 75-83; 10) an E156S substitution; 11) a G166S substitution; 12) a G169A substitution; 13) an S188P substitution; 14) a Q206C substitution; 15) an N212G substitution; 16) a Y217L substitution; 17) an N218S substitution; 18) a T254A substitution; 19) a S271E substitution; 20) a Y104A substitution; and 21) a G128S substitution. The amino acid sequence such a variant is depicted in Figure 7B (SEQ ID NO:9), where the underlined residues 75-83 are deleted.

[0098] In some embodiments, a suitable subtilisin polypeptide is a variant subtilisin and comprises, compared to the amino acid sequence set forth in Figure 6 (SEQ ID NO:7), all of the following changes: 1) a Q2K substitution; 2) an S3C substitution; 3) a P5S substitution; 4) an S9A substitution; 5) an I31L substitution; 6) a K43N substitution; 7) an M50F substitution; 8) an A73L substitution; 9) deletion of amino acids 75-83; 10) an E156S substitution; 11) a G166S substitution; 12) a G169A substitution; 13) an S188P substitution; 14) a Q206C substitution; 15) an N212G substitution; 16) a Y217L substitution; 17) an N218S substitution; 18) a T254A substitution; 19) a S271E substitution; 20) a Y104A substitution; 21) a G128S

substitution; and 22) a D32A substitution. The amino acid sequence such a variant is depicted in Figure 7C (SEQ ID NO:10), where the underlined residues 75-83 are deleted.

[0099] In some embodiments, a suitable subtilisin polypeptide is a variant subtilisin and comprises, compared to the amino acid sequence set forth in Figure 6 (SEQ ID NO:7), all of the following changes: 1) a Q2K substitution; 2) an S3C substitution; 3) a P5S substitution; 4) an S9A substitution; 5) an I31L substitution; 6) a K43N substitution; 7) an M50F substitution; 8) an A73L substitution; 9) deletion of amino acids 75-83; 10) an E156S substitution; 11) a G166S substitution; 12) a G169A substitution; 13) an S188P substitution; 14) a Q206C substitution; 15) an N212G substitution; 16) a Y217L substitution; 17) an N218S substitution; 18) a T254A substitution; 19) a S271E substitution; 20) a Y104A substitution; 21) a G128S substitution; and 22) a D32S substitution. The amino acid sequence such a variant is depicted in Figure 7D (SEQ ID NO:11), where the underlined residues 75-83 are deleted.

[00100] In some embodiments, a suitable subtilisin polypeptide is a variant subtilisin and comprises, compared to the amino acid sequence set forth in Figure 6 (SEQ ID NO:7), all of the following changes: 1) a Q2K substitution; 2) an S3C substitution; 3) a P5S substitution; 4) an S9A substitution; 5) an I31L substitution; 6) a K43N substitution; 7) an M50F substitution; 8) an A73L substitution; 9) deletion of amino acids 75-83; 10) an E156S substitution; 11) a G166S substitution; 12) a G169A substitution; 13) an S188P substitution; 14) a Q206C substitution; 15) an N212G substitution; 16) a Y217L substitution; 17) an N218S substitution; 18) a T254A substitution; 19) a S271E substitution; 20) a Y104A substitution; 21) a G128S substitution; and 22) a D32V substitution. The amino acid sequence such a variant is depicted in Figure 7E (SEQ ID NO:12), where the underlined residues 75-83 are deleted.

[00101] In some embodiments, the subtilisin polypeptide is immobilized on an insoluble support. Any of a variety of standard chemical reactions can be carried out to immobilize a subtilisin polypeptide onto an insoluble support. For example, the insoluble support can include a moiety suitable for attachment of a protein, e.g., a moiety that allows for formation of an amide bond between the moiety and the subtilisin polypeptide. Immobilization of a subtilisin polypeptide onto an insoluble support can be carried out using a carbodiimide amidation reagent. A suitable amidation reagent is N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), which can be supplemented by N-hydroxysuccinimide (NHS) to facilitate the reaction. As another example, where the insoluble support includes a terminal aldehyde group, conjugation can be effected by reductive amination with a suitable reducing reagent, such as sodium cyanoborohydride. Other commonly used cross-linking agents that are suitable for use include, e.g., 1,1-bis(diazoacetyl)-

2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimide yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light, and can also be used. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and reactive substrates are employed for protein immobilization.

[00102] A subtilisin polypeptide can be immobilized directly, or via a linker, where suitable linkers can be of a flexible nature, although other chemical linkages are not excluded. For example suitable linkers include peptides of between about 6 and about 40 amino acids in length, or between about 6 and about 25 amino acids in length. These linkers are generally produced by using synthetic, linker-encoding oligonucleotides to couple the proteins.

Binding

[00103] A composition (also referred to as a "sample") comprising an affinity peptide-containing AAV virion (e.g., an AAV virion comprising a subtilisin prodomain-AAV capsid fusion protein; or a subtilisin prodomain-AAV virion) is contacted with an immobilized subtilisin polypeptide under conditions that promote binding of the subtilisin prodomain-AAV virion to the immobilized subtilisin, but do not promote cleavage of the subtilisin prodomain from a subtilisin prodomain-AAV capsid protein present in the subtilisin prodomain-AAV virion.

[00104] Generally, a sample comprising a subtilisin prodomain-AAV virion is applied to the immobilized subtilisin, and the resin is equilibrated with a solution. "Conditions for binding" include a condition of the sample being applied, as well as any equilibration conditions. Those skilled in the art can readily determine appropriate conditions for binding of a subtilisin prodomain-AAV virion in a sample to an immobilized subtilisin.

[00105] The pH conditions suitable for applying a sample comprising a subtilisin prodomain-AAV virion to an immobilized subtilisin range from about 5.5 to about 8.5, from about 6.0 to about 8.0, from about 7.0 to about 8.0, from about 7.5 to about 8.0, or from about 7.0 to about 7.5. Temperature conditions suitable for applying a sample comprising a subtilisin prodomain-AAV virion to an immobilized subtilisin range from about 15°C to about 37°C, from about 20°C to about 35°C, or from about 22°C to about 25°C. Salt conditions generally range from about 0.01 M to about 1 M, e.g., from about 0.01 M to about 0.05 M, from about 0.05 M to

about 0.1 M, from about 0.1 M to about 0.25 M, from about 0.25 M to about 0.5 M, or from about 0.5 M to about 1 M.

[00106] Various additional substances may be included, including, but not limited to, detergents (e.g., sodium dodecyl sulfate, e.g., from about 0.05% to about 2%); non-ionic detergents, e.g., Tween 20™, and the like; chaotropic agents and denaturants, e.g., urea, and guanidinium HCl; buffers, e.g., Tris-based buffers, borate –based buffers, phosphate-based buffers, imidazole, HEPES, PIPES, MOPS, PIPES, TES, and the like.

[00107] In some embodiments, equilibration conditions and binding conditions include 0.1 M potassium phosphate buffer, pH 7.0-8.0 (e.g., pH 7.2), and a temperature of about 20°C to about 25°C. In other embodiments, equilibration conditions and binding conditions include 100 mM sodium phosphate buffer, 20 mM Tris-HCl, pH 7.0-8.0 (e.g., pH 7.2), and a temperature of about 20°C to about 25°C.

Washing conditions

[00108] One or more washing steps may be included, to remove undesired (e.g., unbound) components. A washing step may be performed after a subtilisin prodomain-AAV virion is bound to an immobilized subtilisin polypeptide. The composition and temperature of a washing solution may vary according to the desired result. The optimal composition and temperature of a washing solution can readily be determined by those skilled in the art, based on known properties of the bound subtilisin prodomain-AAV virion. Wash solutions may comprise a buffer, and may further comprise additional components, as necessary, including, but not limited to, a detergent. In some embodiments, the wash conditions will be the same as the binding conditions.

Elution

[00109] The bound subtilisin prodomain-AAV virion is released upon application of conditions that activate catalytic cleavage of a subtilisin prodomain from a subtilisin prodomain-AAV capsid fusion protein present in the subtilisin prodomain-AAV virion.

[00110] Conditions that activate catalytic cleavage of a subtilisin prodomain from a subtilisin prodomain-AAV capsid fusion protein present in the subtilisin prodomain-AAV virion include, but are not limited to, fluoride ions, chloride ions, and high pH (e.g., pH higher than about 8.0, higher than about 8.5, or higher than about 9.0). Exemplary suitable activation conditions include, e.g., potassium fluoride in a concentration range of from about 10 mM to about 200 mM, e.g., from about 10 mM to about 50 mM, from about 50 mM to about 100 mM, from about 100 mM to about 150 mM, or from about 150 mM to about 200 mM; potassium fluoride

in a concentration range of from about 50 mM to about 1 M, e.g., from about 50 mM to about 100 mM, from about 100 mM to about 500 mM, or from about 500 mM to about 1 M.

[00111] The above-mentioned activation conditions can be added to the binding conditions. For example, activation conditions can include, e.g., 0.1 M KF, 0.1 M potassium phosphate, pH 7.0-8.0 (e.g., pH 7.5), and a temperature of from about 20°C to about 25°C.

AAV capsid proteins

[00112] As noted above, an affinity peptide-containing AAV virion comprises a fusion protein comprising an AAV capsid protein and a heterologous affinity peptide. Amino acid sequences of AAV capsid proteins are known in the art. See, e.g., GenBank Accession No. YP_680427 (AAV-2 VP2); GenBank Accession No. YP_680426 (AAV-2 VP1); GenBank Accession No. YP_068409 (AAV-5 VP1); GenBank Accession No. YP_077178 (AAV-7 VP1); and GenBank Accession No. YP_077180 (AAV-8 VP1).

[00113] In some embodiments, the amino acid sequence of the AAV capsid protein present in the affinity peptide-AAV capsid fusion protein is a wild-type amino acid sequence, e.g., an amino acid sequence naturally found in an AAV capsid protein. In other embodiments, the amino acid sequence of the AAV capsid protein present in the affinity peptide-AAV capsid fusion protein has at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, or at least about 99% amino acid sequence identity to the amino acid sequence depicted in Figure 10.

[00114] In other embodiments, the amino acid sequence of the AAV capsid protein present in the affinity peptide-AAV capsid fusion protein has at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, or at least about 99% amino acid sequence identity to the AAV2 VP2 amino acid sequence depicted in Figure 8 ("AAV2 VP2"; SEQ ID NO:14).

[00115] In other embodiments, the amino acid sequence of the AAV capsid protein comprises from about 1 to about 20 amino acid substitutions compared to the amino acid sequence depicted in Figure 8 ("AAV2 VP2"; SEQ ID NO:14) or Figure 10 (SEQ ID NO:16), e.g., the amino acid sequence of the AAV capsid protein comprises from about 1 to about 5, from about 5 to about 10, from about 10 to about 15, or from about 15 to about 20, amino acid substitutions compared to the amino acid sequence depicted in Figure 8 or Figure 10. In some embodiments, the amino acid substitution(s) provide for one or more of: 1) increased capacity of an AAV virion comprising the capsid protein to cross an endothelial cell layer, compared to an AAV virion comprising a capsid protein without the substitution(s); 2) increased infectivity of a non-permissive cell type (e.g., a hepatocyte, a stem cell, a lung epithelial cell) compared to

an AAV virion comprising a capsid protein without the substitution(s); and 3) decreased binding to neutralizing antibody, e.g., neutralizing antibody present in the serum of a mammal, where decreased binding to neutralizing antibody results in increased infectivity due to avoidance of neutralizing antibody-mediated reduction of infectivity. Suitable amino acid substitutions that provide for the aforementioned properties are described in, e.g., US Patent Publication No. 2005/0052922; and PCT Publication WO 2005/005610, the contents of which are incorporated herein by reference in their entirety.

NUCLEIC ACIDS ENCODING A VARIANT AAV CAPSID PROTEIN

- [00116] The present invention provides nucleic acids (e.g., recombinant nucleic acids) comprising a nucleotide sequence encoding a variant AAV capsid protein, where the variant AAV capsid protein comprises a heterologous affinity peptide. Also provided are expression vectors comprising the nucleic acids; isolated host cells comprising the nucleic acids; and isolated host cells comprising the expression vectors.
- [00117] In the following description, variant AAV-2 capsid proteins or variant AAV-8 capsid proteins are exemplified. However, the exemplification of AAV-2 or AAV-8 herein is in no way meant to be limiting. Those skilled in the art can readily adapt the methods as discussed herein to generate capsid mutants of other AAV, including, e.g., AAV-3, AAV-4, AAV-5, etc.
- [00118] A subject nucleic acid comprises a nucleotide sequence encoding at least one of VP1, VP2, and VP3, wherein at least one of VP1, VP2, and VP3 comprises an affinity peptide. A subject nucleic acid comprises a nucleotide sequence encoding a fusion protein comprising an AAV capsid protein and an affinity peptide, where such fusion proteins are described above.
- [00119] Nucleotide sequences encoding AAV capsid proteins are known in the art. See, e.g., GenBank Accession No. NC_001401 (e.g., nucleotides 2203-4410 of the sequence provided in NC_001401, which is an AAV-2 complete genome sequence); GenBank Accession No. NC_006152 (e.g., nucleotides 2207-4381 of the sequence provided in NC_006152, which is an AAV-5 complete genome sequence); GenBank Accession No. NC_006260 (e.g., nucleotides 2222-4435 of the sequence provided in NC_006260, which is an AAV-7 complete genome sequence); and GenBank Accession No. NC_006261 (e.g., nucleotides 2121-4337 of the sequence provided in NC_006261, which is an AAV-8 complete genome sequence).
- [00120] Nucleotide sequences encoding a subtilisin prodomain as described above include nucleotide sequences that have at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, or at least about 99% identity to the nucleotide sequence depicted in Figure 9 (e.g., nucleotides 183-413 of the sequence set forth in GenBank Accession No. X00165; SEQ ID NO:15).

[00121] Nucleotide sequences encoding a metal ion affinity peptide are readily derived from the amino acid sequences of such peptides.

[00122] In some embodiments, the nucleic acid is present in an expression vector that provides for expression of the encoded affinity peptide-AAV capsid fusion protein, and production of the fusion protein in an appropriate host cell, e.g., a mammalian cell.

[00123] In some embodiments, the nucleic acid further comprises nucleotide sequences encoding an AAV rep protein.

[00124] The present invention further provides host cells, e.g., isolated host cells, comprising a subject nucleic acid. A subject host cell can be an isolated cell, e.g., a cell in *in vitro* culture. A subject host cell is useful for producing an affinity peptide-AAV capsid fusion protein; and can also be used for producing an affinity peptide-AAV virion, as described below. Where a subject host cell is used to produce an affinity peptide-AAV virion, it is referred to as a "packaging cell." In some embodiments, a subject host cell is stably genetically modified with a subject nucleic acid. In other embodiments, a subject host cell is transiently genetically modified with a subject nucleic acid.

[00125] A subject nucleic acid is introduced stably or transiently into a host cell, using established techniques, including, but not limited to, electroporation, calcium phosphate precipitation, liposome-mediated transfection, and the like. For stable transformation, a subject nucleic acid will generally further include a selectable marker, e.g., any of several well-known selectable markers such as neomycin resistance, and the like.

[00126] A subject host cell is generated by introducing a subject nucleic acid into any of a variety of cells, e.g., mammalian cells, including, e.g., murine cells, and primate cells (e.g., human cells). Suitable mammalian cells include, but are not limited to, primary cells and cell lines, where suitable mammalian cell lines include, but are not limited to, HeLa cells (e.g., American Type Culture Collection (ATCC) No. CCL-2), CHO cells (e.g., ATCC Nos. CRL9618, CCL61, CRL9096), 293 cells (e.g., ATCC No. CRL-1573), Vero cells, NIH 3T3 cells (e.g., ATCC No. CRL-1658), Huh-7 cells, BHK cells (e.g., ATCC No. CCL10), PC12 cells (ATCC No. CRL1721), COS cells, COS-7 cells (ATCC No. CRL1651), RAT1 cells, mouse L cells (ATCC No. CCL1.3), human embryonic kidney (HEK) cells (ATCC No. CRL1573), HLHepG2 cells, and the like.

[00127] In some embodiments, a subject host cell includes, in addition to a nucleic acid comprising a nucleotide sequence encoding a mutant capsid protein, a nucleic acid that comprises a nucleotide sequence encoding one or more AAV rep proteins. In other embodiments, a subject host cell further comprises an rAAV vector, as described below. As

described in more detail below, in some embodiments, an rAAV virion is generated using a subject host cell.

rAAV VIRIONS

[00128] A subject variant capsid protein can be incorporated into an AAV virion that comprises a heterologous nucleic acid that provides for production of a heterologous gene product (e.g., a heterologous nucleic acid or a heterologous protein). A subject recombinant AAV virion ("rAAV virion") comprises a subject variant capsid protein, and includes a heterologous nucleic acid that comprises a nucleotide sequence encoding a heterologous gene product. Thus, the present invention provides rAAV virions that comprise a subject variant capsid protein, as described above; and a heterologous nucleic acid. A subject rAAV virion is infectious (e.g., can infect one or more cells or cell types in an individual), and is thus useful for introducing a gene product into an individual.

[00129] In some embodiments, a subject rAAV virion comprises an affinity peptide-AAV capsid fusion protein that comprises a metal ion affinity peptide. In these embodiments, the metal ion affinity peptide is present in the rAAV virion in both unpurified (e.g., cell lysate) and purified states.

[00130] In other embodiments, a subject rAAV virion comprises an affinity peptide-AAV capsid fusion protein that comprises a protease substrate, e.g., a subtilisin prodomain. In these embodiments, the protease substrate is present in the rAAV virion in the unpurified state, but is cleaved from the rAAV virion during the purification process, and is not present in the purified rAAV virion.

Additional properties

[00131] As noted above, in some embodiments, an affinity peptide-AAV capsid fusion protein present in an AAV virion can comprises one or more amino acid substitution(s) that provide for one or more of: 1) increased capacity of an AAV virion comprising the capsid protein to cross an endothelial cell layer, compared to an AAV virion comprising a capsid protein without the substitution(s); 2) increased infectivity of a non-permissive cell type (e.g., a hepatocyte, a stem cell, a lung epithelial cell) compared to an AAV virion comprising a capsid protein without the substitution(s); and 3) decreased binding to neutralizing antibody, e.g., neutralizing antibody present in the serum of a mammal, where decreased binding to neutralizing antibody results in increased infectivity due to avoidance of neutralizing antibody-mediated reduction of infectivity. Suitable amino acid substitutions that provide for the aforementioned properties are described in, e.g., US Patent Publication No. 2005/0052922; and

PCT Publication WO 2005/005610, the contents of which are incorporated herein by reference in their entirety.

[00132] In some embodiments, a subject rAAV virion exhibits increased resistance to neutralizing antibodies compared to wild-type AAV or AAV comprising a wild-type capsid protein. In these embodiments, a subject rAAV virion has from about 10-fold to about 10,000-fold greater resistance to neutralizing antibodies than wt AAV, e.g., a subject rAAV virion has from about 10-fold to about 25-fold, from about 25-fold to about 50-fold, from about 50-fold to about 75-fold, from about 75-fold to about 100-fold, from about 100-fold to about 150-fold, from about 150-fold to about 200-fold, from about 200-fold to about 250-fold, from about 250-fold to about 300-fold, at least about 350-fold, at least about 400-fold, from about 400-fold to about 450-fold, from about 450-fold to about 500-fold, from about 500-fold to about 550-fold, from about 550-fold to about 600-fold, from about 600-fold to about 700-fold, from about 700-fold to about 800-fold, from about 800-fold to about 900-fold, from about 900-fold to about 1000-fold, from about 1,000-fold to about 2,000-fold, from about 2,000-fold to about 3,000-fold, from about 3,000-fold to about 4,000-fold, from about 4,000-fold to about 5,000-fold, from about 5,000-fold to about 6,000-fold, from about 6,000-fold to about 7,000-fold, from about 7,000-fold to about 8,000-fold, from about 8,000-fold to about 9,000-fold, or from about 9,000-fold to about 10,000-fold greater resistance to neutralizing antibodies than a wild-type AAV or an AAV comprising a wild-type capsid protein.

[00133] In some embodiments, a subject rAAV virion exhibits decreased binding to a neutralizing antibody that binds a wild-type AAV capsid protein. For example, a subject mutant rAAV virion exhibits from about 10-fold to about 10,000-fold reduced binding to a neutralizing antibody that binds a wild-type AAV capsid protein. For example, a subject mutant rAAV virion exhibits from about 10-fold to about 25-fold, from about 25-fold to about 50-fold, from about 50-fold to about 75-fold, from about 75-fold to about 100-fold, from about 100-fold to about 150-fold, from about 150-fold to about 200-fold, from about 200-fold to about 250-fold, from about 250-fold to about 300-fold, at least about 350-fold, at least about 400-fold, from about 400-fold to about 450-fold, from about 450-fold to about 500-fold, from about 500-fold to about 550-fold, from about 550-fold to about 600-fold, from about 600-fold to about 700-fold, from about 700-fold to about 800-fold, from about 800-fold to about 900-fold, from about 900-fold to about 1000-fold, from about 1,000-fold to about 2,000-fold, from about 2,000-fold to about 3,000-fold, from about 3,000-fold to about 4,000-fold, from about 4,000-fold to about 5,000-fold, from about 5,000-fold to about 6,000-fold, from about 6,000-fold to about 7,000-fold, from about 7,000-fold to about 8,000-fold, from about 8,000-fold to

about 9,000-fold, or from about 9,000-fold to about 10,000-fold reduced binding to a neutralizing antibody that binds a wild-type capsid AAV protein, compared to the binding affinity of the antibody to wild-type AAV capsid protein.

[00134] In some embodiments, an anti-AAV neutralizing antibody binds to a subject rAAV virion with an affinity of less than about 10^{-7} M, less than about 5×10^{-6} M, less than about 10^{-6} M, less than about 5×10^{-5} M, less than about 10^{-5} M, less than about 10^{-4} M, or lower.

[00135] A subject rAAV virion that exhibits reduced binding to neutralizing antibodies has increased residence time in the body, compared to the residence time of an AAV virion comprising wild-type capsid proteins. Thus, e.g., a subject rAAV virion has at least about 25%, at least about 50%, at least about 75%, at least about 2-fold, at least about 2.5-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold at least about 10-fold, at least about 15-fold, at least about 20-fold, at least about 50-fold, or more, increased residence time *in vivo* compared to the residence time of an AAV virion comprising wild-type capsid proteins.

[00136] Whether a given mutant rAAV virion exhibits reduced binding to a neutralizing antibody and/or increased resistance to neutralizing antibody can be determined using any known assay, including the assay described in the Example. For example, mutant rAAV virion is contacted with a permissive cell type, e.g., 293 cells, in the presence of neutralizing antibody. A control sample contains the cells, mutant rAAV virion, and no neutralizing antibody. After a suitable time, the cells are contacted with adenovirus, and rAAV particles are detected. The level of rAAV particles is compared to the amount of rAAV particles that are generated in the absence of neutralizing antibody.

[00137] In some embodiments, a subject rAAV virion exhibits increased ability to infect a cell that is relatively refractory to AAV infection (e.g., a non-permissive cell). In these embodiments, a subject mutant AAV exhibits at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 2-fold, at least about 4-fold, at least about 10-fold, at least about 20-fold, or at least about 50-fold, or more, greater infectivity of a non-permissive cell than a wild-type AAV or an rAAV virion comprising wild-type capsid protein.

[00138] Examples of cells that are relatively refractory to AAV infection include, but are not limited to stem cells, hepatocytes, and lung epithelial cells.

[00139] The term "stem cell" is used herein to refer to a mammalian cell that has the ability both to self-renew, and to generate differentiated progeny (see, e.g., Morrison et al. (1997) *Cell* 88:287-298). Generally, stem cells also have one or more of the following properties: an ability to undergo asynchronous, or symmetric replication, that is where the two daughter cells after

division can have different phenotypes; extensive self-renewal capacity; capacity for existence in a mitotically quiescent form; and clonal regeneration of all the tissue in which they exist, for example the ability of hematopoietic stem cells to reconstitute all hematopoietic lineages.

"Progenitor cells" differ from stem cells in that they typically do not have the extensive self-renewal capacity, and often can only regenerate a subset of the lineages in the tissue from which they derive, for example only lymphoid, or erythroid lineages in a hematopoietic setting.

[00140] Stem cells may be characterized by both the presence of markers associated with specific epitopes identified by antibodies and the absence of certain markers as identified by the lack of binding of specific antibodies. Stem cells may also be identified by functional assays both in vitro and in vivo, particularly assays relating to the ability of stem cells to give rise to multiple differentiated progeny.

[00141] Stem cells of interest include hematopoietic stem cells and progenitor cells derived therefrom (U.S. Pat. No. 5,061,620); neural crest stem cells (see Morrison et al. (1999) Cell 96:737-749); adult neural stem cells and neural progenitor cells; embryonic stem cells; mesenchymal stem cells; mesodermal stem cells; induced pluripotent stem (iPS) cells; etc. Other hematopoietic "progenitor" cells of interest include cells dedicated to lymphoid lineages, e.g. immature T cell and B cell populations.

[00142] In some embodiments, a subject rAAV virion exhibits increased ability to cross an endothelial cell layer. For example, in these embodiments, a subject rAAV virion exhibits at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 2-fold, at least about 5-fold, at least about 10-fold, at least about 25-fold, or at least about 50-fold increase in ability to cross an endothelial cell layer.

[00143] Whether a given rAAV virion exhibits an increased ability to cross an endothelial cell layer can be determined experimentally using well-known systems.

Generation of subject rAAV virions

[00144] By way of introduction, it is typical to employ a host or "producer" cell for rAAV vector replication and packaging. Such a producer cell (usually a mammalian host cell) generally comprises or is modified to comprise several different types of components for rAAV production. The first component is a recombinant adeno-associated viral (rAAV) vector genome (or "rAAV pro-vector") that can be replicated and packaged into vector particles by the host packaging cell. The rAAV pro-vector will normally comprise a heterologous polynucleotide (or "transgene"), with which it is desired to genetically alter another cell in the context of gene therapy (since the packaging of such a transgene into rAAV vector particles

can be effectively used to deliver the transgene to a variety of mammalian cells). The transgene is generally flanked by two AAV inverted terminal repeats (ITRs) which comprise sequences that are recognized during excision, replication and packaging of the AAV vector, as well as during integration of the vector into a host cell genome.

[00145] A second component is a helper virus that can provide helper functions for AAV replication. Although adenovirus is commonly employed, other helper viruses can also be used as is known in the art. Alternatively, the requisite helper virus functions can be isolated genetically from a helper virus and the encoding genes can be used to provide helper virus functions in trans. The AAV vector elements and the helper virus (or helper virus functions) can be introduced into the host cell either simultaneously or sequentially in any order.

[00146] The final components for AAV production to be provided in the producer cell are "AAV packaging genes" such as AAV rep and cap genes that provide replication and encapsidation proteins, respectively. Several different versions of AAV packaging genes can be provided (including rep-cap cassettes and separate rep and/or cap cassettes in which the rep and/or cap genes can be left under the control of the native promoters or operably linked to heterologous promoters. Such AAV packaging genes can be introduced either transiently or stably into the host packaging cell, as is known in the art and described in more detail below. Variant AAV capsid proteins (e.g., affinity peptide-AAV capsid fusion proteins) are discussed in detail above.

1. rAAV vector

[00147] A subject rAAV virion, including the heterologous DNA of interest (where "heterologous DNA of interest" is also referred to herein as "heterologous nucleic acid"), can be produced using standard methodology, known to those of skill in the art. The methods generally involve the steps of (1) introducing a subject rAAV vector into a host cell; (2) introducing an AAV helper construct into the host cell, where the helper construct includes AAV coding regions capable of being expressed in the host cell to complement AAV helper functions missing from the AAV vector; (3) introducing one or more helper viruses and/or accessory function vectors into the host cell, wherein the helper virus and/or accessory function vectors provide accessory functions capable of supporting efficient recombinant AAV ("rAAV") virion production in the host cell; and (4) culturing the host cell to produce rAAV virions. The AAV expression vector, AAV helper construct and the helper virus or accessory function vector(s) can be introduced into the host cell, either simultaneously or serially, using standard transfection techniques.

- [00148]** AAV expression vectors are constructed using known techniques to at least provide as operatively linked components in the direction of transcription, control elements including a transcriptional initiation region, the DNA of interest and a transcriptional termination region. The control elements are selected to be functional in a mammalian muscle cell. The resulting construct which contains the operatively linked components is bounded (5' and 3') with functional AAV ITR sequences.
- [00149]** The nucleotide sequences of AAV ITR regions are known. See, e.g., Kotin, R. M. (1994) Human Gene Therapy 5:793-801; Berns, K. I. "Parvoviridae and their Replication" in Fundamental Virology, 2nd Edition, (B. N. Fields and D. M. Knipe, eds.) for the AAV-2 sequence. AAV ITRs used in the vectors of the invention need not have a wild-type nucleotide sequence, and may be altered, e.g., by the insertion, deletion or substitution of nucleotides. Additionally, AAV ITRs may be derived from any of several AAV serotypes, including without limitation, AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-7, etc. Furthermore, 5' and 3' ITRs which flank a selected nucleotide sequence in an AAV expression vector need not necessarily be identical or derived from the same AAV serotype or isolate, so long as they function as intended, i.e., to allow for excision and rescue of the sequence of interest from a host cell genome or vector, and to allow integration of the DNA molecule into the recipient cell genome when AAV Rep gene products are present in the cell. ITRs allow replication of the vector sequence in the presence of an appropriate mixture of Rep proteins. ITRs also allow for the incorporation of the vector sequence into the capsid to generate an AAV particle.
- [00150]** A suitable heterologous DNA molecule (also referred to herein as a "heterologous nucleic acid") for use in a subject rAAV vector will generally be less than about 5 kilobases (kb) in size and will include, for example, a gene (a nucleotide sequence) that encodes a protein that is defective or missing from a recipient subject; a gene that encodes a protein having a desired biological or therapeutic effect (e.g., an antibacterial, antiviral or antitumor function); a nucleotide sequence that encodes an RNA that inhibits or reduces production of a deleterious or otherwise undesired protein; a nucleotide sequence that encodes an antigenic protein; or a nucleotide sequence that encodes an RNA that inhibits or reduces production of a protein.
- [00151]** Suitable heterologous nucleic acids include, but are not limited to, those encoding proteins used for the treatment of endocrine, metabolic, hematologic, cardiovascular, neurologic, musculoskeletal, urologic, pulmonary and immune disorders, including such disorders as inflammatory diseases, autoimmune, chronic and infectious diseases, such as acquired immunodeficiency syndrome (AIDS), cancer, hypercholesterolemia, insulin disorders

such as diabetes, growth disorders, various blood disorders including various anemias, thalassemias and hemophilia; genetic defects such as cystic fibrosis, Gaucher's Disease, Hurler's Disease, adenosine deaminase (ADA) deficiency, emphysema, or the like.

[00152] Suitable heterologous nucleic acids include, but are not limited to, those encoding any of a variety of proteins, including, but not limited to: a cytokine, a chemokine, an angiogenesis-inducing polypeptide, an apoptosis-inducing polypeptide, an angiogenesis-inhibiting polypeptide, an antibody, a growth factor, a polypeptide that induced cell differentiation, a polypeptide that increases cell proliferation, a colony stimulating factor, a blood clotting factor, and the like.

[00153] Suitable heterologous nucleic acids include, but are not limited to, those encoding any of a variety of proteins, including, but not limited to: an interferon (e.g., IFN- γ , IFN- α , IFN- β , IFN- ω , IFN- τ); an insulin (e.g., Novolin, Humulin, Humalog, Lantus, Ultralente, etc.); an erythropoietin ("EPO"; e.g., Procrit®, Eprex®, or Epogen® (epoetin- α); Aranesp® (darbepoietin- α); NeoRecormon®, Epogin® (epoetin- β); and the like); an antibody (e.g., a monoclonal antibody) (e.g., Rituxan® (rituximab); Remicade® (infliximab); Herceptin® (trastuzumab); Humira™ (adalimumab); Xolair® (omalizumab); Bexxar® (tositumomab); Raptiva™ (efalizumab); Erbitux™ (cetuximab); and the like), including an antigen-binding fragment of a monoclonal antibody; a blood factor (e.g., Activase® (alteplase) tissue plasminogen activator; NovoSeven® (recombinant human factor VIIa); Factor VIIa; Factor VIII (e.g., Kogenate®); Factor IX; β -globin; hemoglobin; and the like); a colony stimulating factor (e.g., Neupogen® (filgrastim; G-CSF); Neulasta (pegfilgrastim); granulocyte colony stimulating factor (G-CSF), granulocyte-monocyte colony stimulating factor, macrophage colony stimulating factor, megakaryocyte colony stimulating factor; and the like); a growth hormone (e.g., a somatotropin, e.g., Genotropin®, Nutropin®, Norditropin®, Saizen®, Serostim®, Humatrope®, etc.; a human growth hormone; and the like); an interleukin (e.g., IL-1; IL-2, including, e.g., Proleukin®; IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9; etc.); a growth factor (e.g., Regranex® (beclapernin; PDGF); Fiblast® (trafermin; bFGF); Stemgen® (ancestim; stem cell factor); keratinocyte growth factor; an acidic fibroblast growth factor, a stem cell factor, a basic fibroblast growth factor, a hepatocyte growth factor; and the like); a soluble receptor (e.g., a TNF- α -binding soluble receptor such as Enbrel® (etanercept); a soluble VEGF receptor; a soluble interleukin receptor; a soluble γ/δ T cell receptor; and the like); an enzyme (e.g., α -glucosidase; Cerazyme® (imiglucarase; β -glucocerebrosidase, Ceredase® (alglucerase;); an enzyme activator (e.g., tissue plasminogen activator); a chemokine (e.g., IP-10; Mig; Gro α /IL-8, RANTES; MIP-1 α ; MIP-1 β ; MCP-1; PF-4; and the

like); an angiogenic agent (e.g., vascular endothelial growth factor (VEGF) ; an anti-angiogenic agent (e.g., a soluble VEGF receptor); a protein vaccine; a neuroactive peptide such as bradykinin, cholecystokinin, gastrin, secretin, oxytocin, gonadotropin-releasing hormone, beta-endorphin, enkephalin, substance P, somatostatin, prolactin, galanin, growth hormone-releasing hormone, bombesin, dynorphin, neurotensin, motilin, thyrotropin, neuropeptide Y, luteinizing hormone, calcitonin, insulin, glucagon, vasopressin, angiotensin II, thyrotropin-releasing hormone, vasoactive intestinal peptide, a sleep peptide, etc.; other proteins such as a thrombolytic agent, an atrial natriuretic peptide, bone morphogenic protein, thrombopoietin, relaxin, glial fibrillary acidic protein, follicle stimulating hormone, a human alpha-1 antitrypsin, a leukemia inhibitory factor, a transforming growth factor, an insulin-like growth factor, a luteinizing hormone, a macrophage activating factor, tumor necrosis factor, a neutrophil chemotactic factor, a nerve growth factor a tissue inhibitor of metalloproteinases; a vasoactive intestinal peptide, angiogenin, angiotropin, fibrin; hirudin; a leukemia inhibitory factor; an IL-1 receptor antagonist (e.g., Kineret® (anakinra)); an ion channel, e.g., cystic fibrosis transmembrane conductance regulator (CFTR); dystrophin; utrophin, a tumor suppressor; lysosomal enzyme acid α -glucosidase (GAA); and the like. Suitable nucleic acids also include those that encode a functional fragment of any of the aforementioned proteins; and nucleic acids that encode functional variants of any of the aforementioned proteins.

[00154] Suitable heterologous nucleic acids also include those that encode antigenic proteins.

A subject rAAV that comprises a heterologous nucleic acid that encodes an antigenic protein is suitable for stimulating an immune response to the antigenic protein in a mammalian host. The antigenic protein is derived from an autoantigen, an allergen, a tumor-associated antigen, a pathogenic virus, a pathogenic bacterium, a pathogenic protozoan, a pathogenic helminth, or any other pathogenic organism that infects a mammalian host. As used herein, the term “a nucleic acid encoding an antigenic protein derived from” includes nucleic acids encoding wild-type antigenic proteins, e.g., a nucleic acid isolated from a pathogenic virus that encodes a viral protein; synthetic nucleic acids generated in the laboratory that encode antigenic proteins that are identical in amino acid sequence to a naturally-occurring antigenic protein; synthetic nucleic acids generated in the laboratory that encode antigenic proteins that differ in amino acid sequence (e.g., by from one amino acid to about 15 amino acids) from a naturally-occurring antigenic protein, but that nonetheless induce an immune response to the corresponding naturally-occurring antigenic protein; synthetic nucleic acids generated in the laboratory that encode fragments of antigenic proteins (e.g., fragments of from about 5 amino acids to about 50 amino acids, which fragments comprises one or more antigenic epitopes),

which fragments induce an immune response to the corresponding naturally-occurring antigenic protein; etc.

[00155] Similarly, an antigenic protein “derived from” an autoantigen, an allergen, a tumor-associated antigen, a pathogenic virus, a pathogenic bacterium, a pathogenic protozoan, a pathogenic helminth, or any other pathogenic organism that infects a mammalian host, includes proteins that are identical in amino acid sequence to a naturally-occurring antigenic protein, and proteins that differ in amino acid sequence (e.g., by from one amino acid to about 15 amino acids) from a naturally-occurring antigenic protein, but that nonetheless induce an immune response to the corresponding naturally-occurring antigenic protein; and fragments of antigenic proteins (e.g., fragments of from about 5 amino acids to about 50 amino acids, which fragments comprises one or more antigenic epitopes), which fragments induce an immune response to the corresponding naturally-occurring antigenic protein.

[00156] In some embodiments, an immune response to an antigenic protein encoded by a subject rAAV will stimulate a protective immune response to a pathogenic organism that displays the antigenic protein or antigenic epitope (or a protein or an epitope that is cross-reactive with the rAAV-encoded antigenic protein or antigenic epitopes) in the mammalian host. In some embodiments, a cytotoxic T lymphocyte (CTL) response to the rAAV-encoded antigenic protein will be induced in the mammalian host. In other embodiments, a humoral response to the rAAV-encoded antigenic protein will be induced in the mammalian host, such that antibodies specific to the antigenic protein are generated. In many embodiments, a TH1 immune response to the rAAV-encoded antigenic protein will be induced in the mammalian host. Suitable antigenic proteins include tumor-associated antigens, viral antigens, bacterial antigens, and protozoal antigens; and antigenic fragments thereof. In some embodiments, the antigenic protein is derived from an intracellular pathogen. In other embodiments, the antigenic protein is a self-antigen. In yet other embodiments, the antigenic protein is an allergen.

[00157] Tumor-specific antigens include, but are not limited to, any of the various MAGEs (Melanoma-Associated Antigen E), including MAGE 1 (e.g., GenBank Accession No. M77481), MAGE 2 (e.g., GenBank Accession No. U03735), MAGE 3, MAGE 4, etc.; any of the various tyrosinases; mutant ras; mutant p53 (e.g., GenBank Accession No. X54156 and AA494311); and p97 melanoma antigen (e.g., GenBank Accession No. M12154). Other tumor-specific antigens include the Ras peptide and p53 peptide associated with advanced cancers, the HPV 16/18 and E6/E7 antigens associated with cervical cancers, MUC11-KLH antigen associated with breast carcinoma (e.g., GenBank Accession No. J03651), CEA (carcinoembryonic antigen) associated with colorectal cancer (e.g., GenBank Accession No.

X98311), gp100 (e.g., GenBank Accession No. S73003) or MART1 antigens associated with melanoma, and the PSA antigen associated with prostate cancer (e.g., GenBank Accession No. X14810). The p53 gene sequence is known (See e.g., Harris et al. (1986) Mol. Cell. Biol., 6:4650-4656) and is deposited with GenBank under Accession No. M14694. Thus, the present invention can be used as immunotherapeutics for cancers including, but not limited to, cervical, breast, colorectal, prostate, lung cancers, and for melanomas.

[00158] Viral antigens are derived from known causative agents responsible for diseases including, but not limited to, measles, mumps, rubella, poliomyelitis, hepatitis A, B (e.g., GenBank Accession No. E02707), and C (e.g., GenBank Accession No. E06890), as well as other hepatitis viruses, influenza, adenovirus (e.g., types 4 and 7), rabies (e.g., GenBank Accession No. M34678), yellow fever, Japanese encephalitis (e.g., GenBank Accession No. E07883), dengue (e.g., GenBank Accession No. M24444), hantavirus, and human immunodeficiency virus (e.g., GenBank Accession No. U18552).

[00159] Suitable bacterial and parasitic antigens include those derived from known causative agents responsible for diseases including, but not limited to, diphtheria, pertussis (e.g., GenBank Accession No. M35274), tetanus (e.g., GenBank Accession No. M64353), tuberculosis, bacterial and fungal pneumonias (e.g., *Haemophilus influenzae*, *Pneumocystis carinii*, etc.), cholera, typhoid, plague, shigellosis, salmonellosis (e.g., GenBank Accession No. L03833), Legionnaire's Disease, Lyme disease (e.g., GenBank Accession No. U59487), malaria (e.g., GenBank Accession No. X53832), hookworm, onchocerciasis (e.g., GenBank Accession No. M27807), schistosomiasis (e.g., GenBank Accession No. L08198), trypanosomiasis, leishmaniasis, giardiasis (e.g., GenBank Accession No. M33641), amoebiasis, filariasis (e.g., GenBank Accession No. J03266), borreliosis, and trichinosis.

[00160] Suitable heterologous nucleic acids that encode heterologous gene products include non-translated RNAs, such as an antisense RNA, a ribozyme, an RNAi and an siRNA. Interfering RNA (RNAi) fragments, particularly double-stranded (ds) RNAi, can be used to inhibit gene expression. One approach well known in the art for inhibiting gene expression is short interfering RNA (siRNA) mediated gene silencing, where the level of expression product of a target gene is reduced by specific double stranded siRNA nucleotide sequences that are complementary to at least a 19-25 nucleotide long segment (e.g., a 20-21 nucleotide sequence) of the target gene transcript, including the 5' untranslated (UT) region, the ORF, or the 3' UT region. In some embodiments, short interfering RNAs are about 19-25 nt in length. See, e.g., PCT applications WO0/44895, WO99/32619, WO01/75164, WO01/92513, WO01/29058, WO01/89304, WO02/16620, and WO02/29858; and U.S. Patent Publication No. 20040023390

for descriptions of siRNA technology. The siRNA can be encoded by a nucleic acid sequence, and the nucleic acid sequence can also include a promoter. The nucleic acid sequence can also include a polyadenylation signal. In some embodiments, the polyadenylation signal is a synthetic minimal polyadenylation signal.

[00161] Target genes include any gene encoding a target gene product (RNA or protein) that is deleterious (e.g., pathological); e.g., a target gene product that is malfunctioning, a target gene product that, when produced in a cell, has a pathological effect, etc. Target gene products include, but are not limited to, huntingtin; hepatitis C virus; human immunodeficiency virus; amyloid precursor protein; tau; a protein that includes a polyglutamine repeat; a herpes virus (e.g., varicella zoster); any pathological virus; and the like.

[00162] As such a subject rAAV that includes a heterologous nucleic acid encoding an siRNA is useful for treating a variety of disorders and conditions, including, but not limited to, neurodegenerative diseases, e.g., a trinucleotide-repeat disease, such as a disease associated with polyglutamine repeats, e.g., Huntington's disease, spinocerebellar ataxia, spinal and bulbar muscular atrophy (SBMA), dentatorubropallidoluysian atrophy (DRPLA), etc.; an acquired pathology (e.g., a disease or syndrome manifested by an abnormal physiological, biochemical, cellular, structural, or molecular biological state) such as a viral infection, e.g., hepatitis that occurs or may occur as a result of an HCV infection, acquired immunodeficiency syndrome, which occurs as a result of an HIV infection; and the like.

[00163] In many embodiments, a heterologous nucleic acid encoding an siRNA is operably linked to a promoter. Suitable promoters are known those skilled in the art and include the promoter of any protein-encoding gene, e.g., an endogenously regulated gene or a constitutively expressed gene. For example, the promoters of genes regulated by cellular physiological events, e.g., heat shock, oxygen levels and/or carbon monoxide levels, e.g., in hypoxia, may be operably linked to an siRNA-encoding nucleic acid.

[00164] The selected heterologous nucleotide sequence, such as EPO-encoding or nucleic acid of interest, is operably linked to control elements that direct the transcription or expression thereof in the nucleotide sequence *in vivo*. Such control elements can comprise control sequences normally associated with the selected gene (e.g., endogenous cellular control elements). Alternatively, heterologous control sequences can be employed. Useful heterologous control sequences generally include those derived from sequences encoding mammalian or viral genes. Examples include, but are not limited to, the SV40 early promoter, mouse mammary tumor virus long terminal repeat (LTR) promoter; adenovirus major late promoter (Ad MLP); a herpes simplex virus (HSV) promoter, an endogenous cellular promoter

that is heterologous to the gene of interest, a cytomegalovirus (CMV) promoter such as the CMV immediate early promoter region (CMVIE), a rous sarcoma virus (RSV) promoter, synthetic promoters, hybrid promoters, and the like. In addition, sequences derived from nonviral genes, such as the murine metallothionein gene, will also find use herein. Such promoter sequences are commercially available from, e.g., Stratagene (San Diego, Calif.).

[00165] In some embodiments, cell type-specific or tissue-specific promoter will be operably linked to the heterologous nucleic acid encoding the heterologous gene product, such that the gene product is produced selectively or preferentially in a particular cell type(s) or tissue(s). In some embodiments, an inducible promoter will be operably linked to the heterologous nucleic acid.

[00166] For example, muscle-specific and inducible promoters, enhancers and the like, are useful for delivery of a gene product to a muscle cell. Such control elements include, but are not limited to, those derived from the actin and myosin gene families, such as from the myoD gene family; the myocyte-specific enhancer binding factor MEF-2; control elements derived from the human skeletal actin gene and the cardiac actin gene; muscle creatine kinase sequence elements and the murine creatine kinase enhancer (mCK) element; control elements derived from the skeletal fast-twitch troponin C gene, the slow-twitch cardiac troponin C gene and the slow-twitch troponin I gene; hypoxia-inducible nuclear factors; steroid-inducible elements and promoters, such as the glucocorticoid response element (GRE); the fusion consensus element for RU486 induction; and elements that provide for tetracycline regulated gene expression.

[00167] The AAV expression vector which harbors the DNA molecule of interest (the heterologous DNA) bounded by AAV ITRs, can be constructed by directly inserting the selected sequence(s) into an AAV genome which has had the major AAV open reading frames ("ORFs") excised therefrom. Other portions of the AAV genome can also be deleted, so long as a sufficient portion of the ITRs remain to allow for replication and packaging functions. Such constructs can be designed using techniques well known in the art. See, e.g., U.S. Pat. Nos. 5,173,414 and 5,139,941; International Publication Nos. WO 92/01070 (published Jan. 23, 1992) and WO 93/03769 (published March 4, 1993); Lebkowski et al. (1988) *Molec. Cell. Biol.* 8:3988-3996; Vincent et al. (1990) *Vaccines 90* (Cold Spring Harbor Laboratory Press); Carter, B. J. (1992) *Current Opinion in Biotechnology* 3:533-539; Muzyczka, N. (1992) *Current Topics in Microbiol. and Immunol.* 158:97-129; Kotin, R. M. (1994) *Human Gene Therapy* 5:793-801; Shelling and Smith (1994) *Gene Therapy* 1:165-169; and Zhou et al. (1994) *J. Exp. Med.* 179:1867-1875.

[00168] Alternatively, AAV ITRs can be excised from the viral genome or from an AAV vector containing the same and fused 5' and 3' of a selected nucleic acid construct that is present in another vector using standard ligation techniques, such as those described in Sambrook et al., supra. For example, ligations can be accomplished in 20 mM Tris-Cl pH 7.5, 10 mM MgCl₂, 10 mM DTT, 33 µg/ml BSA, 10 mM-50 mM NaCl, and either 40 µM ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0 °C to 16° C (for "sticky end" ligation) or 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14° C (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 30-100 µg/ml total DNA concentrations (5-100 nM total end concentration). AAV vectors which contain ITRs have been described in, e.g., U.S. Pat. No. 5,139,941. In particular, several AAV vectors are described therein which are available from the American Type Culture Collection ("ATCC") under Accession Numbers 53222, 53223, 53224, 53225 and 53226.

[00169] Additionally, chimeric genes can be produced synthetically to include AAV ITR sequences arranged 5' and 3' of one or more selected nucleic acid sequences. Preferred codons for expression of the chimeric gene sequence in mammalian muscle cells can be used. The complete chimeric sequence is assembled from overlapping oligonucleotides prepared by standard methods. See, e.g., Edge, Nature (1981) 292:756; Nambair et al. Science (1984) 223:1299; Jay et al. J. Biol. Chem. (1984) 259:6311.

[00170] In order to produce rAAV virions, an AAV expression vector is introduced into a suitable host cell using known techniques, such as by transfection. A number of transfection techniques are generally known in the art. See, e.g., Graham et al. (1973) Virology, 52:456, Sambrook et al. (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratories, New York, Davis et al. (1986) Basic Methods in Molecular Biology, Elsevier, and Chu et al. (1981) Gene 13:197. Particularly suitable transfection methods include calcium phosphate co-precipitation (Graham et al. (1973) Virol. 52:456-467), direct micro-injection into cultured cells (Capecchi, M. R. (1980) Cell 22:479-488), electroporation (Shigekawa et al. (1988) BioTechniques 6:742-751), liposome mediated gene transfer (Mannino et al. (1988) BioTechniques 6:682-690), lipid-mediated transduction (Felgner et al. (1987) Proc. Natl. Acad. Sci. USA 84:7413-7417), and nucleic acid delivery using high-velocity microprojectiles (Klein et al. (1987) Nature 327:70-73).

[00171] For the purposes of the invention, suitable host cells for producing rAAV virions include microorganisms, yeast cells, insect cells, and mammalian cells, that can be, or have been, used as recipients of a heterologous DNA molecule. The term includes the progeny of the original cell which has been transfected. Thus, a "host cell" as used herein generally refers

to a cell which has been transfected with an exogenous DNA sequence. Cells from the stable human cell line, 293 (readily available through, e.g., the American Type Culture Collection under Accession Number ATCC CRL1573) are used in many embodiments. Particularly, the human cell line 293 is a human embryonic kidney cell line that has been transformed with adenovirus type-5 DNA fragments (Graham et al. (1977) *J. Gen. Virol.* 36:59), and expresses the adenoviral E1a and E1b genes (Aiello et al. (1979) *Virology* 94:460). The 293 cell line is readily transfected, and provides a particularly convenient platform in which to produce rAAV virions.

2. AAV Helper Functions

[00172] Host cells containing the above-described AAV expression vectors must be rendered capable of providing AAV helper functions in order to replicate and encapsidate the nucleotide sequences flanked by the AAV ITRs to produce rAAV virions. AAV helper functions are generally AAV-derived coding sequences which can be expressed to provide AAV gene products that, in turn, function in trans for productive AAV replication. AAV helper functions are used herein to complement necessary AAV functions that are missing from the AAV expression vectors. Thus, AAV helper functions include one, or both of the major AAV ORFs, namely the rep and cap coding regions, or functional homologues thereof. In the context of the instant invention, the cap functions include one or more mutant capsid proteins, wherein at least one capsid protein comprises at least one mutation, as described above.

[00173] By "AAV rep coding region" is meant the art-recognized region of the AAV genome which encodes the replication proteins Rep 78, Rep 68, Rep 52 and Rep 40. These Rep expression products have been shown to possess many functions, including recognition, binding and nicking of the AAV origin of DNA replication, DNA helicase activity and modulation of transcription from AAV (or other heterologous) promoters. The Rep expression products are collectively required for replicating the AAV genome. For a description of the AAV rep coding region, see, e.g., Muzyczka, N. (1992) *Current Topics in Microbiol. and Immunol.* 158:97-129; and Kotin, R. M. (1994) *Human Gene Therapy* 5:793-801. Suitable homologues of the AAV rep coding region include the human herpesvirus 6 (HHV-6) rep gene which is also known to mediate AAV-2 DNA replication (Thomson et al. (1994) *Virology* 204:304-311).

[00174] AAV cap proteins include VP1, VP2, and VP3, where AAV capsid proteins are as described above. For example, an AAV capsid protein can be a fusion protein comprising an AAV capsid protein and an affinity peptide, as described above; and can further include one or more amino acid substitutions that provide for one or more of increased capacity to cross an

endothelial cell layer, increased infectivity of a non-permissive cell, and decreased binding to a neutralizing antibody.

[00175] AAV helper functions are introduced into the host cell by transfecting the host cell with an AAV helper construct either prior to, or concurrently with, the transfection of the AAV expression vector. AAV helper constructs are thus used to provide at least transient expression of AAV rep and/or cap genes to complement missing AAV functions that are necessary for productive AAV infection. AAV helper constructs lack AAV ITRs and can neither replicate nor package themselves. These constructs can be in the form of a plasmid, phage, transposon, cosmid, virus, or virion. A number of AAV helper constructs have been described, such as the commonly used plasmids pAAV/Ad and pIM29+45 which encode both Rep and Cap expression products. See, e.g., Samulski et al. (1989) J. Virol. 63:3822-3828; and McCarty et al. (1991) J. Virol. 65:2936-2945. A number of other vectors have been described which encode Rep and/or Cap expression products. See, e.g., U.S. Pat. No. 5,139,941.

[00176] Both AAV expression vectors and AAV helper constructs can be constructed to contain one or more optional selectable markers. Suitable markers include genes which confer antibiotic resistance or sensitivity to, impart color to, or change the antigenic characteristics of those cells which have been transfected with a nucleic acid construct containing the selectable marker when the cells are grown in an appropriate selective medium. Several selectable marker genes that are useful in the practice of the invention include the hygromycin B resistance gene (encoding Aminoglycoside phosphotransferase (APH)) that allows selection in mammalian cells by conferring resistance to hygromycin; the neomycin phosphotransferase gene (encoding neomycin phosphotransferase) that allows selection in mammalian cells by conferring resistance to G418; and the like. Other suitable markers are known to those of skill in the art.

3. AAV Accessory Functions

[00177] The host cell (or packaging cell) must also be rendered capable of providing non AAV derived functions, or "accessory functions," in order to produce rAAV virions. Accessory functions are non AAV derived viral and/or cellular functions upon which AAV is dependent for its replication. Thus, accessory functions include at least those non AAV proteins and RNAs that are required in AAV replication, including those involved in activation of AAV gene transcription, stage specific AAV mRNA splicing, AAV DNA replication, synthesis of Cap expression products and AAV capsid assembly. Viral-based accessory functions can be derived from any of the known helper viruses.

[00178] Particularly, accessory functions can be introduced into and then expressed in host cells using methods known to those of skill in the art. Commonly, accessory functions are provided

by infection of the host cells with an unrelated helper virus. A number of suitable helper viruses are known, including adenoviruses; herpesviruses such as herpes simplex virus types 1 and 2; and vaccinia viruses. Nonviral accessory functions will also find use herein, such as those provided by cell synchronization using any of various known agents. See, e.g., Buller et al. (1981) *J. Virol.* 40:241-247; McPherson et al. (1985) *Virology* 147:217-222; Schlehofer et al. (1986) *Virology* 152:110-117.

[00179] Alternatively, accessory functions can be provided using an accessory function vector. Accessory function vectors include nucleotide sequences that provide one or more accessory functions. An accessory function vector is capable of being introduced into a suitable host cell in order to support efficient AAV virion production in the host cell. Accessory function vectors can be in the form of a plasmid, phage, transposon, cosmid, or another virus. Accessory vectors can also be in the form of one or more linearized DNA or RNA fragments which, when associated with the appropriate control elements and enzymes, can be transcribed or expressed in a host cell to provide accessory functions.

[00180] Nucleic acid sequences providing the accessory functions can be obtained from natural sources, such as from the genome of an adenovirus particle, or constructed using recombinant or synthetic methods known in the art. In this regard, adenovirus-derived accessory functions have been widely studied, and a number of adenovirus genes involved in accessory functions have been identified and partially characterized. See, e.g., Carter, B. J. (1990) "Adeno-Associated Virus Helper Functions," in *CRC Handbook of Parvoviruses*, vol. I (P. Tijssen, ed.), and Muzyczka, N. (1992) *Curr. Topics. Microbiol. and Immun.* 158:97-129. Specifically, early adenoviral gene regions E1a, E2a, E4, VAI RNA and, possibly, E1b are thought to participate in the accessory process. Janik et al. (1981) *Proc. Natl. Acad. Sci. USA* 78:1925-1929. Herpesvirus-derived accessory functions have been described. See, e.g., Young et al. (1979) *Prog. Med. Virol.* 25:113. Vaccinia virus-derived accessory functions have also been described. See, e.g., Carter, B. J. (1990), *supra.*, Schlehofer et al. (1986) *Virology* 152:110-117.

[00181] As a consequence of the infection of the host cell with a helper virus, or transfection of the host cell with an accessory function vector, accessory functions are expressed which transactivate the AAV helper construct to produce AAV Rep and/or Cap proteins. The Rep expression products excise the recombinant DNA (including the DNA of interest, e.g., the heterologous nucleic acid) from the AAV expression vector. The Rep proteins also serve to duplicate the AAV genome. The expressed Cap proteins assemble into capsids, and the

recombinant AAV genome is packaged into the capsids. Thus, productive AAV replication ensues, and the DNA is packaged into rAAV virions.

[00182] Following recombinant AAV replication, rAAV virions can be purified as described above.

[00183] The resulting rAAV virions are then ready for use for DNA delivery, such as in gene therapy applications, or for the delivery of a gene product to a mammalian host.

EXAMPLES

[00184] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Celsius, and pressure is at or near atmospheric. Standard abbreviations may be used, e.g., bp, base pair(s); kb, kilobase(s); pl, picoliter(s); s or sec, second(s); min, minute(s); h or hr, hour(s); aa, amino acid(s); kb, kilobase(s); bp, base pair(s); nt, nucleotide(s); i.m., intramuscular(ly); i.p., intraperitoneal(ly); s.c., subcutaneous(ly); and the like.

Example 1: Production of AAV virions including a histidine-tagged capsid protein

MATERIALS AND METHODS

Construction of AAV plasmids:

[00185] To construct pXX2Not, a 1.8-kilobase linear fragment containing a portion backbone sequence of pXX2 (Xiao *et al.*, (1998) J Virol 72, 2224-2232) was generated by PCR using the primers 5'-GCGAAGCTTACGCGGCCGCTTGTTAATCAATAAACCGTTTAATTTCG-3' (SEQ ID NO:29) and 5'-CGGAATGGACGATATCCCGC-3' (SEQ ID NO:30) with pXX2 (Xiao *et al.*, (1998) *supra*) as template. Both this product and pXX2 were digested with *Hind III* and *Cla I*, and the products were ligated to create the rAAV packaging plasmid, pXX2Not. The *cap* sequence from AAV8 was cloned into this vector as well. To construct each peptide mutant, spliced overlap extension PCR was used to insert the His₆ peptide epitopes into the correct location, sometimes with additional flanking sequences. Primer sequences and cloning details are available upon request. In addition, an AAV2 library containing a randomly

inserted His₆ sequence within the *cap* gene was packaged (Maheshri *et al.* (2006) Nat Biotechnol 24, 198-204) and selected as previously described (Yu and Schaffer (2006) J Virol 80, 3285-3292). Briefly, the chloroamphenicol resistance (Cam^R) gene was randomly inserted into a plasmid containing the AAV2 *cap* gene by using a transposon kit (Finnyzmes, Espoo, Finland). The resulting plasmid library was digested to excise the *cap*-CamR genes, which were subsequently cloned into pSub2 (Maheshri *et al.*, (2006) *supra*). The pSub2 plasmid library was digested with *Not I* before ligation to His₆ fragments phosphorylated with T4 polynucleotide kinase. The His₆ insert was constructed using the following oligonucleotides, with the histidine codons shown in bold: 5'- GGCCGGTC**ACCACCACCACC**ACTC-3' (SEQ ID NO:31) and 5'- GGCCGAGT**GGTGGTGGTGGTGGT**GACC-3' (SEQ ID NO:32).

Cell lines and vector production:

[00186] HEK293T, HeLa, CHO K1, CHO pgsA, and CHO pgsD were cultured in Iscove's modified Dulbecco's medium (IMDM) (Mediatech, Herndon, VA) with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1% penicillin and streptomycin (Invitrogen) at 37°C and 5% CO₂. All cell lines were obtained from the ATCC. AAV293 cells (Stratagene) were cultured in Dulbecco's modified medium (DMEM) with 10% fetal bovine serum and 1% penicillin and streptomycin at 37°C and 5% CO₂.

[00187] rAAV was produced as previously described (Kaspar *et al.*, (2003) Science 301, 839-842; Maheshri *et al.*, (2006) *supra*). Briefly, in a ~80% confluent 15 cm plate of AAV293 (Stratagene) cells, ~15 µg pAAV CMV-GFP, 15 µg of pHelper (Stratagene), and 15 µg of one of the modified pXX2Not plasmids were transfected by the calcium phosphate method. Viral vectors were harvested as previously described (Maheshri *et al.*, 2006). Vector packaged in wild type capsid was purified via an iodixanol gradient followed by heparin column chromatography (Zolotukhin *et al.*, (1999) Gene Ther 6, 973-985). DNase-resistant genomic titers were determined using quantitative PCR, and infectious titers were determined using flow cytometry as previously described (Maheshri *et al.*, (2006) *supra*).

Ni-NTA Purification of Viral Vectors:

[00188] A mixture of 1 volume of cell lysate containing virus, 0.5 volume binding buffer (10mM Tris pH 8.0, 300mM NaCl, and 20mM imidazole), and 500 µL of 50% Ni-NTA agarose (Qiagen, Valencia, CA) were agitated gently overnight at 4°C. This mixture was then loaded onto a plastic column (Kontes, Vineland, NJ) before washing with 5 mL of wash buffer (10mM Tris pH 8.0, 50mM imidazole) and eluting with 2-3 mL of elution buffer (10mM Tris pH 8.0, 500mM imidazole). Finally, the eluted virus was buffer exchanged into TBS and

concentrated using Microcon spin columns (Millipore, Billerica, MA) according to the manufacturer's instructions.

[00189] Equivalent amounts of each column sample from a large scale purification scheme were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were detected by using a silver stain kit (Bio-Rad, Hercules, CA). The DMEM and viral cell lysate samples analyzed on the same gel were diluted 1:10 to prevent oversaturation of the silver stain signal.

[00190] For Western blot analysis, equivalent amounts of GFP vector packaged with WT AAV2 and His₆ capsids were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose blot. The blot was blocked in Tris-buffered saline with 3% bovine serum albumin and incubated with penta-his antibody (Qiagen) according to the manufacturer's instructions. The blots were then developed by the ECL detection assay (Amersham Biosciences, Piscataway, NJ).

***In Vitro* Characterization of Viral Vectors:**

[00191] To assay for heparin binding, $\sim 10^{11}$ purified genomic particles of virus were loaded onto a 1-mL HiTrap heparin column (Amersham) previously equilibrated with 150 mM NaCl and 50 mM Tris at pH 7.5. The virus was eluted using 0.75 mL volumes of 50 mM Tris buffer containing increasing increments of 50 mM NaCl up to 750 mM, followed by two 1 M washes. To quantify the amount of infectious virus present in each fraction, 75 μ L of each elution fraction was added to 2.5×10^5 293 cells in 12-well format. At 48 hours post-infection, the fraction of green cells was quantified by flow cytometry at the U.C. Berkeley Cancer Center (Beckman-Coulter EPICS).

[00192] To compare cell tropism, either wild-type or mutant rAAV-GFP particles were added to 293T, HeLa, CHO K1, CHO pgsA, and CHO pgsD cell lines at a genomic MOI of 1000. After 48 hours, the fraction of green cells was quantified by flow cytometry at the U.C. Berkeley Cancer Center (Beckman-Coulter EPICS). Antibody neutralization assays using human intravenous immunoglobulin (IvIg) (Bayer) were performed as previously described (Maheshri *et al.*, (2006) *supra*).

Animal Surgeries and Histology:

[00193] Recombinant AAV vectors were stereotactically injected into the striatum region of the brain (AP, +0.2; ML, $\pm$ 3.5; DV, -4.5 from skull) of adult female Fischer 344 rats (150 g, 6 weeks). The animals were deeply anesthetized with a mixture of ketamine (90 mg/kg animal) and xylazine (10 mg/kg animal) prior to injection, and 3 μ L of high-titer AAV vectors (1×10^9 vg/ μ L) were injected using Hamilton syringe. Animals were transcardially perfused with 4%

paraformaldehyde in PBS, and the brains were excised after either at 3 days and 3 weeks post-injection, for detecting immune responses ($n = 2$ per condition) and quantifying GFP expression ($n = 3$ per condition), respectively. The retrieved brains were post-fixed by immersing in 4% PFA overnight at 4 °C and subsequently stored in 30% sucrose for cryoprotection prior to sectioning.

[00194] Coronal sections were cut with 40 μm thickness using a freezing, sliding microtome. Primary mouse-anti ED1 (1:100, Chemicon) and mouse-anti OX8 (1:100, Chemicon) were utilized to detect antigens expressed by macrophages and T-cells, respectively. Additionally, primary mouse-anti NeuN (1:200, Chemicon) and guinea pig-anti GFAP (1:1000, Advanced ImmunoChemical) were utilized to identify cell types (i.e., neurons and glial cells), and GFP expression was amplified using primary rabbit anti-GFP (1:2000, Invitrogen). Corresponding secondary antibodies (labeled with AlexaFluor 488, 546, and 633) were used for detection. For nucleus staining, some sections were counterstained using To-PRO-3 (1:2000, Invitrogen). The sections containing regions exhibiting GFP expression were collected, and the total volume of GFP expression was quantified using a modified stereology method (Yu and Schaffer, 2006). Statistical comparisons between wt AAV and His₆ AAV injections were performed using the ANOVA t-test (JMP software, SAS Institute Inc.). Animal protocols were approved by the UCB Animal Care and Use Committee and conducted in accordance with NIH guidelines.

RESULTS

Construction of AAV mutants containing His₆ tag

[00195] A library of *cap*-Cam^R insertion mutants was constructed using a MuA Transposase and a modified transposon insert containing a chloroamphenicol resistance gene (Cam^R). The resulting *cap*-CAM^R library was cloned into the AAV vector pSub2, and the Cam^R gene was subsequently replaced with a His₆-encoding insert to create the final library. Five nucleotides from *cap* become duplicated at the insertion site during the transposition reaction, and these together with the His₆ (SEQ ID NO:38) sequence yield a 13 amino acid (aa) insertion. Restriction digest analysis of individual clones from the library confirmed that the insertions occurred throughout the entire *cap* gene and that each of these clones contained only one insert. The estimated diversity of the library was $\sim 5 \times 10^4$ independent insertions into the 2.6-kb *cap* PCR product, which thus likely provides full coverage of all possible insertion sites. The library was significantly diluted and used to package replication competent AAV (rcAAV), as previously described for AAV library packaging (Maheshri *et al.*, (2006) *supra*).

[00196] While the library likely contains every insertion site, the flanking residues for the His₆ tag, which consist of four alanine residues along with three random amino acids, may decrease

infectivity due to the large insertion size or may not be optimal for display of the His₆ tag. In parallel, a series of defined *cap*-His₆ insertion mutants was constructed using spliced overlap extension PCR. These AAV2 insertion sites, previously shown to be permissive to the insertion of peptide sequences, were located in three regions: immediately after aa position 584 (Shi *et al.*, 2001), after aa position 587 (Girod *et al.*, 1999), and after aa position 588 (Grifman *et al.*, 2001) (Fig. 1A). In addition, an AAV8 mutant containing the peptide insertion in an analogous loop, after aa position 590, was constructed. Each insert contained a His₆ sequence flanked by leucine and glycine on the N-terminal side and serine on the other to aid in efficient peptide display (Fig. 1B). These mutant *cap* variants were inserted into a modified AAV helper plasmid, pXX2Not, based on pXX2 (Xiao *et al.*, (1998) J Virol 72, 2224-2232).

[00197] **FIGURE 1.** Design of His₆ AAV Mutants. (A) Map of VP3 monomer, constructed with Rasmol, indicating where insertions of His₆ tag occurred. (B) Sequences of the insertion site of WT AAV2 (LQRGNRQA; SEQ ID NO:33) compared to sequences at the insertion sites of each of the His₆ AAV2 clones along with the His₆ AAV8 clone. His₆ tags are shown in bold and flanking residues are shown in italics. The sequences of the His₆ clones at the insertion sites are: His584: LQLGHHHHHHSRGNRQA (SEQ ID NO:34); His587: LQRGNLGHHHHHHSRQA (SEQ ID NO:35); His588: LQRGNRLGHHHHHHSQA (SEQ ID NO:36); HisAAV8: QQQNLGHHHHHHSTAP (SEQ ID NO:37).

Analysis of Packaging and Ni-NTA Binding Ability of AAV Clones

[00198] To select the *cap*-His₆ random insertion library for variants capable of binding to Ni-NTA resin, while retaining viral infectivity, the viral library was passed through a Ni-NTA column. The virus present in the elution fractions was then amplified by a low multiplicity of infection (MOI) of 293T cells, followed by the addition of adenovirus serotype 5 (Ad5) to induce replication of the infectious AAV variants, which were again applied to the Ni-NTA column. After only two such rounds of selection, sequence analysis of the library indicated that the pool contained one dominant clone with an insertion after aa position 454, which bound to the Ni-NTA column at levels greatly exceeding that of WT AAV2. The resulting His₆-*cap* gene, along with a corresponding His₆-*cap* gene in which the His₆ was rationally inserted at position 454 with the same leucine, glycine, and serine flanking amino acids used in the other defined insertion clones, were then inserted into pXX2Not. These constructs along with the other rationally designed His₆ clones were then used to package rAAV-GFP.

[00199] Initial characterization of both the library-derived His₆-AAV clone and rationally designed His₆-AAV variants surprisingly indicated that only one (His587) produced high titer, infectious virus (>10⁵ IU/mL). Initial, small scale production on a 10-cm plate yielded ~4x10⁶

IU/mL in clarified cell lysate for this His₆ mutant compared to $\sim 3 \times 10^7$ IU/mL for WT AAV2 capsid. Surprisingly, the remaining AAV2 clones, including the variant with the insertion at aa 454 identified from the viral library, packaged virus with poor infectivity, i.e. with genome/infectious ratios of $>10^5$. For example, optimization of the transposition reaction, which incorporates an additional 5 amino acids onto each peptide insertion, may require the use of alternate transposases or extensive mutagenesis work to remove the extraneous nucleotides or the addition several rounds of amplification prior to selection, despite the fact that the analogous approach worked very effectively in the generation of His-tagged vesicular stomatitis virus protein for retroviral and lentiviral purification (Yu and Schaffer, (2006) *supra*). It is possible that the need for viral proteins to assemble into a 60-mer capsid, rather than a trimer envelope protein, place additional constraints on the insert location and sequence.

[00200] Importantly, the AAV2 variant with a His₆ tag inserted after amino acid 587 (His₆ AAV2), when applied to the Ni-NTA column in clarified cell lysate, bound at levels vastly exceeding that of WT AAV2 (Fig. 2A and B). Further optimization of incubation times, buffer pH, wash conditions, and elution conditions using the His₆ AAV2 clone resulted in a total infectious virus recovery exceeding 90% in a relatively small volume (~ 1 -2 mL). Interestingly, only a moderate 10% of the total DNase-resistant AAV2 viral genomes were recovered from the column, indicating the presence of a large population of non-infectious, DNase-resistant genomes in the crude lysate that were unable to bind to the column. This result is consistent with prior peptide insertions into this region of the AAV2 capsid (Wu *et al.*, (2000) J Virol 74, 8635-8647; Shi *et al.*, (2001) Hum Gene Ther 12, 1697-1711).

[00201] In parallel, the His₆ AAV8 variant, containing an identical peptide insertion after aa 590, showed no significant difference in viral production levels. Remarkably, upon application of His₆ AAV8 to the NiNTA column, over 90% of the both the DNase-resistant viral genomes and infectious virions were recovered from the column (Fig. 2A and B), in marked contrast to the His₆ AAV2 results.

[00202] The purification was scaled up, and a high titer rAAV2-GFP was generated by the direct application of clarified crude lysate to a column packed with Ni-NTA resin and elution of the bound virus with high concentrations of imidazole. High titer rAAV2-GFP with the WT AAV2 capsid was also generated by a conventional two step purification (i.e. centrifugation through an iodixanol gradient followed by flow through a heparin column) (Zolotukhin *et al.*, (1999) Gene Ther 6, 973-985). To assess viral purity, SDS-PAGE analysis followed by silver staining was conducted on the IMAC column purified virus compared to conventional iodixanol and heparin column purified rAAV2-GFP (Zolotukhin *et al.*, (1999) *supra*). The

result indicated a negligible difference between the purities of the final viral stocks, and Western blot analysis confirmed the presence of the His₆ tag within all three viral capsid proteins.

[00203] FIGURE 2. Ni-NTA Purification of His₆ AAV mutant. (A) Representative genomic titers of recombinant GFP vector packaged inside WT AAV2 capsid, His₆ AAV2, and His₆ AAV8 present within each column fraction. Titters were determined using quantitative PCR, and error bars represent the standard deviation of three independent trials. (B) Representative transduction titers of recombinant GFP vector packaged with WT AAV2 capsid, His₆ AAV2, and His₆ AAV8 present within each column fraction. Titters were determined using flow cytometry, and error bars represent the standard deviation of three independent trials.

***In Vitro* Characterization of His₆-tagged AAV**

[00204] Since the His₆ insertion occurs within a region of the AAV2 capsid important for heparan sulfate binding (Kern *et al.*, (2003) J Virol 77, 11072-11081; Opie *et al.*, (2003) J Virol 77, 6995-7006), the properties of His₆ AAV2 were analyzed in vitro. Both purified His₆ AAV2 and vector packaged in wild type capsid were loaded onto a heparin column and eluted with a series of increasing salt concentrations, and infectious viral titers within each fraction were determined by flow cytometry. WT AAV2 eluted in a sharp peak from 400 mM-500 mM NaCl, consistent with previous reports (Clark *et al.*, (1999) Hum Gene Ther 10, 1031-1039; Maheshri *et al.*, (2006) *supra*), whereas His₆ AAV2 eluted in a broad range, beginning at 200 mM NaCl followed by a long tail stretching to 750 mM NaCl, indicating a mixed population of infectious virions with variable heparin affinities (Fig. 3A).

[00205] In addition, several cell lines possessing various levels of heparan sulfate were employed to analyze the cell tropism of the mutant relative to WT AAV2 (Fig. 3B). Infection of 293T and HeLa cell lines showed a modest 2- to 4-fold reduction in the ratio of infectious to genome titers of His₆ AAV2, respectively, compared to WT AAV2. However, this difference increased to almost 10-fold on the native CHO-K1 cell line and two mutant cell lines, CHO pgsA and pgsD, defective in HSPG biosynthesis (Esko *et al.*, (1985) Proc Natl Acad Sci USA 82, 3197-3201), indicating that the mutant was more sensitive to lower HSPG levels, likely due to the disruption of heparan sulfate binding region on the viral capsid. Previous reports indicated that peptide insertion or a single point mutation within this region conferred moderate resistance to antibody neutralization (Huttner *et al.*, (2003) Gene Ther 10, 2139-2147; Maheshri *et al.*, (2006) *supra*). Consistent with these findings, the His₆ insertion interestingly conferred the viral vector with ~6-fold improvement in gene delivery relative to WT AAV2 in the presence of pooled intravenous human immunoglobulin (IvIg) (Fig. 3C).

[00206] FIGURE 3. In vitro characterization of His₆ AAV gene delivery properties. (A) Heparin column chromatograms for WT AAV2 and His₆ AAV2. (B) Representative transduction titers of recombinant GFP vector packaged by WT AAV2 and His₆ AAV2 on several cell lines expressing varying levels of HSPG and representative transduction titer of WT AAV8 and His₆ AAV8 on 293T. Error bars represent the standard deviation from studies performed in biological triplicate. (C) Relative gene delivery efficiency of WT AAV2 and His₆ AAV2 in the presence of varying amounts of human intravenous immunoglobulin. Error bars represent the standard deviation from studies performed in biological triplicate.

***In Vivo* Performance of AAV Vectors**

[00207] Vector purification methods should yield viral vectors with high in vivo biological activity, while minimizing any immunogenic response arising from contaminants. IMAC purified His₆ AAV2 and iodixanol/heparin column purified AAV2 (1x10⁹ DNase-resistant particles/μL), carrying cDNA encoding GFP driven from a human CMV promoter, were stereotactically injected in the rat striatum. Two weeks post-injection, robust GFP expression was observed in all animals, and the cellular tropism of His₆ AAV2 remained identical to that of vector packaged with wild type capsid, with infection primarily of cells staining positive for the neuronal marker NeuN with minimal apparent infection of cells expressing the astrocytic marker glial fibrillary acidic protein (GFAP). Interestingly, a small number of neurons exhibited GFP expression adjacent to the needle track only three days after injection of both vectors. In addition, there was no statistically significant difference in the viral infection spread along the anterior-posterior axis or the total volume accessed by viral vector infection between the two viral preparations (Figs. 4A and 4B), indicating that the His₆ AAV2 variant is highly efficient in vivo and that its performance is not substantially affected by altered heparin affinity. A potential concern for gene therapy is the development of a host immune response against the injected viral vector (Manno *et al.*, (2006) Nat Med 12, 342-347). Three days post-injection, minimal activation of either CD8-positive T lymphocytes or macrophages was observed with either viral vector. Control injections with PBS exhibited similar low activation levels of macrophages and very few CD8-positive T lymphocytes.

[00208] FIGURE 4. In vivo comparisons between injections of GFP-expressing vectors with either WT AAV2 or His₆ AAV2 capsid: GFP expression after 3 weeks. (A) The anterior-posterior (AP) spread of GFP expression indicates no significant difference between the two vectors (P>0.05). The AP spread was the distance between the first and last tissue sections exhibiting GFP expression. (B) The total volume of GFP expression (P>0.05). The error bars for (A), (B) indicate the standard deviations.

Example 2: Production of rAAV virions comprising an altered capsid protein with a protease substrate

[00209] A fusion protein comprising a subtilisin prodomain and an AAV2 VP2 capsid protein was generated. The amino acid sequence of the subtilisin prodomain-AAV2 VP2 capsid protein is shown in Figure 8 (SEQ ID NO:13). An AAV virion comprising this fusion protein bound to immobilized subtilisin, and cleavage of the subtilisin prodomain from the AAV virion was activated by potassium fluoride. No substantial reduction in infectivity of the subtilisin prodomain-AAV virion, compared to an AAV virion with wild-type AAV2 capsid protein, was observed. Figure 12 provides viral production titer from the parent AAV2 and the AAV2 variant ("PalAAV2") with the subtilisin tag attached to the end of the viral protein VP2. Figure 13 provides an infectious elution profile from the subtilisin affinity column. The parent AAV2 does not adhere to the column effectively; PalAAV2 adheres to the subtilisin column in a manner such that it can be effectively recovered.

[00210] While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

CLAIMS

What is claimed is:

1. A method for purification of an adeno-associated virus (AAV) virion, the method comprising:
 - a) contacting a composition comprising an AAV virion with a binding moiety, wherein the AAV virion comprises a variant capsid protein that comprises an affinity peptide, wherein the affinity peptide binds to the binding moiety, forming a bound AAV virion; and
 - b) collecting the bound AAV virion.
2. The method of claim 1, wherein the binding moiety is immobilized on an insoluble support.
3. The method of claim 1, further comprising washing any unbound components present in the composition from the bound AAV virion.
4. The method of claim 1, wherein the collected AAV virion is at least 90% pure.
5. The method of claim 1, wherein the collected AAV virion is infectious.
6. The method of claim 1, wherein the affinity peptide is a metal ion affinity peptide, and wherein the binding moiety is a metal ion.
7. The method of claim 6, wherein the metal ion is immobilized on an insoluble support, and wherein said collecting step comprises eluting the bound AAV virion.
8. The method of claim 1, wherein the affinity peptide is a protease substrate, and wherein the binding moiety is a protease.
9. The method of claim 8, wherein the protease substrate is a subtilisin prodomain peptide, and wherein the protease is subtilisin.

10. The method of claim 9, wherein the subtilisin is immobilized on an insoluble support, and wherein said collecting step comprises activation of protease activity.

11. The method of claim 10, wherein said activation comprises contacting the bound AAV virion with a protease activation trigger selected from high pH, fluoride ions, and chloride ions.

12. The method of claim 1, wherein the composition is a cell lysate.

13. A nucleic acid comprising a nucleotide sequence encoding a variant adeno-associated virus (AAV) capsid protein, wherein the variant AAV capsid protein comprises a heterologous affinity peptide.

14. The nucleic acid of claim 13, wherein the heterologous affinity peptide is a metal ion affinity peptide.

15. The nucleic acid of claim 13, wherein the heterologous affinity peptide is a protease substrate.

16. The nucleic acid of claim 15, wherein the protease substrate is a subtilisin prodomain.

17. An isolated host cell comprising the nucleic acid of claim 13.

18. The isolated host cell of claim 17, wherein the host cell is stably transfected with the nucleic acid.

19. The isolated host cell of claim 17, further comprising a nucleic acid comprising a nucleotide sequence encoding an AAV rep protein.

20. The isolated host cell of claim 19, further comprising a recombinant AAV vector.

21. A recombinant adeno-associated virus (rAAV) virion comprising a variant capsid protein and an rAAV vector comprising a heterologous nucleic acid, wherein the variant capsid protein comprises a heterologous affinity peptide.

22. The rAAV virion of claim 21, wherein the affinity peptide is a metal ion affinity peptide.

23. The rAAV virion of claim 21, wherein the affinity peptide is protease substrate.

24. The rAAV virion of claim 21, wherein the heterologous nucleic acid comprises a nucleotide sequence encoding a gene product selected from a protein and an interfering nucleic acid.

25. A composition comprising the rAAV virion of claim 21, wherein the rAAV virion is at least 90% pure.

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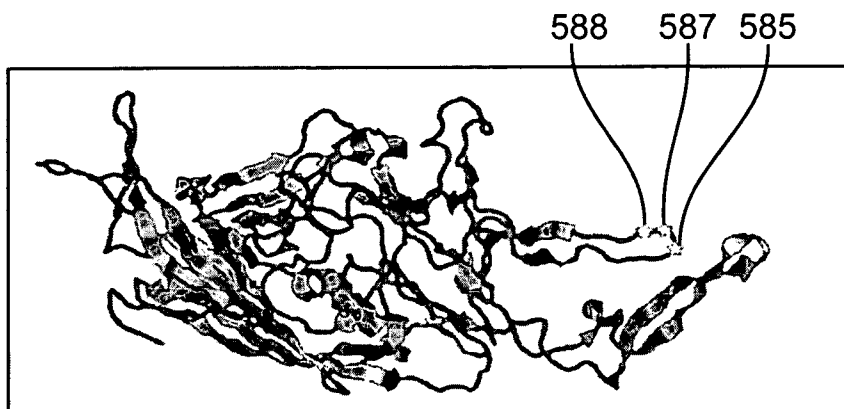


FIG. 1A

Location	Amino Acid Sequence
WT AA V2	~583 LQRGNRQA~ SEQ ID NO: 33
His584	~LQLGHHHHHHSRGNRQA~ SEQ ID NO: 34
His587	~LQRGNLGHHHHHHSRQA~ SEQ ID NO: 35
His588	~LQRGNRLGHHHHHHSQA~ SEQ ID NO: 36
HisAA V8	~QQQNLGHHHHHHSTAP~ SEQ ID NO: 37

FIG. 1B

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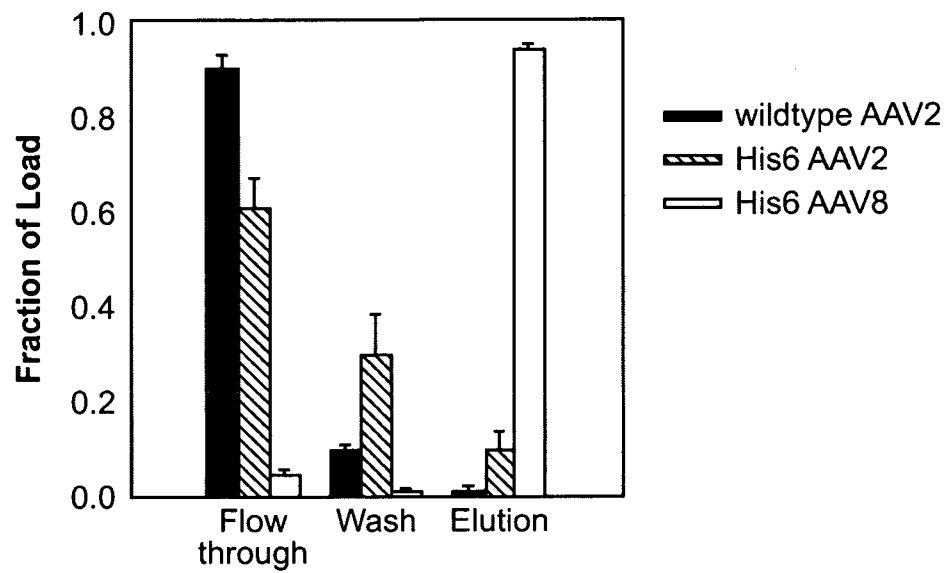


FIG. 2A

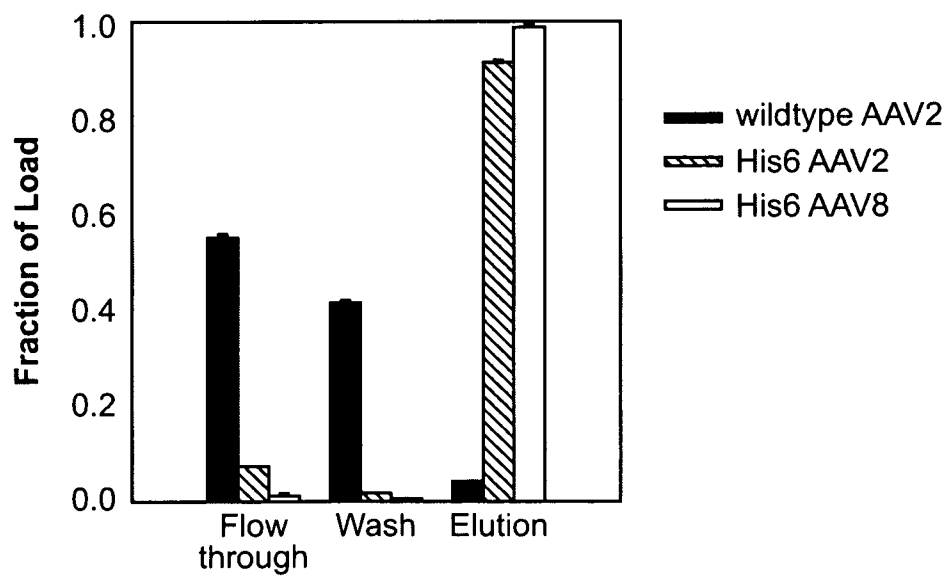


FIG. 2B

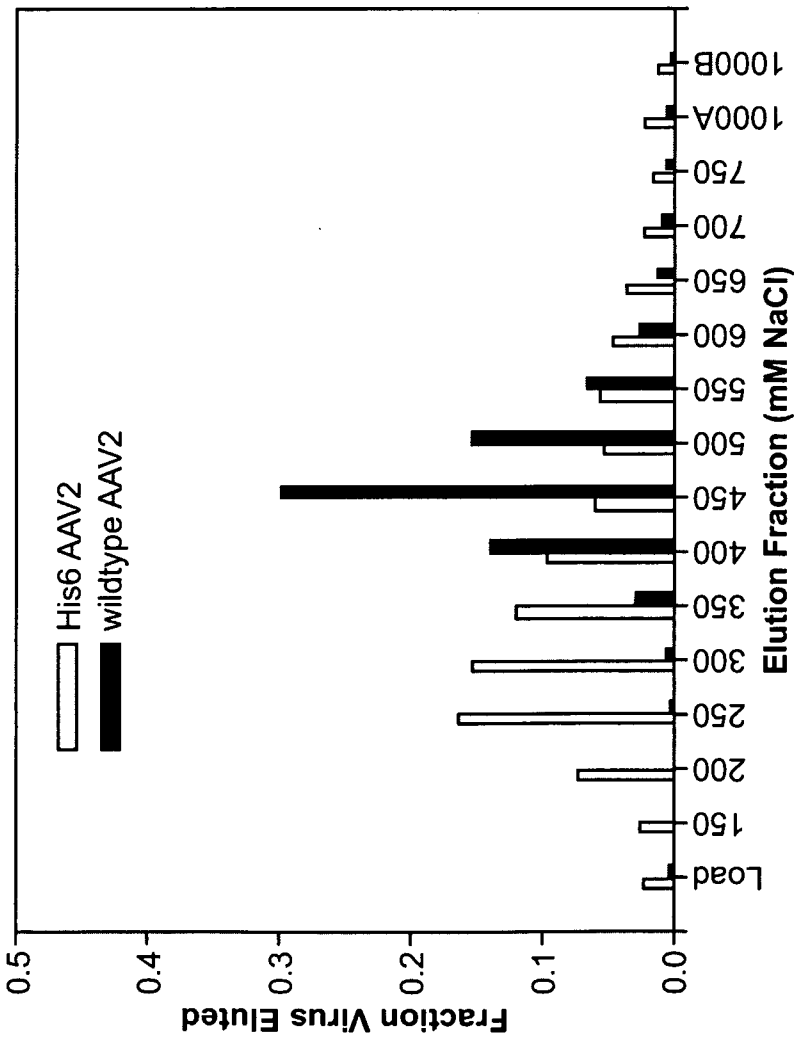


FIG. 3A

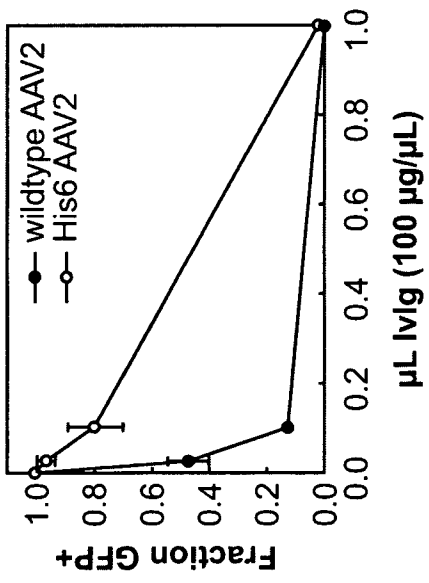


FIG. 3C

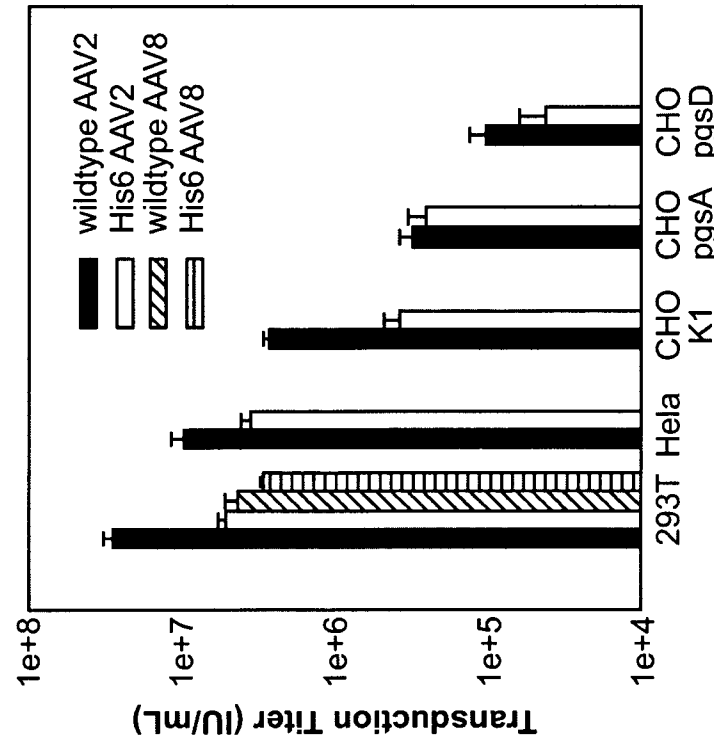


FIG. 3B

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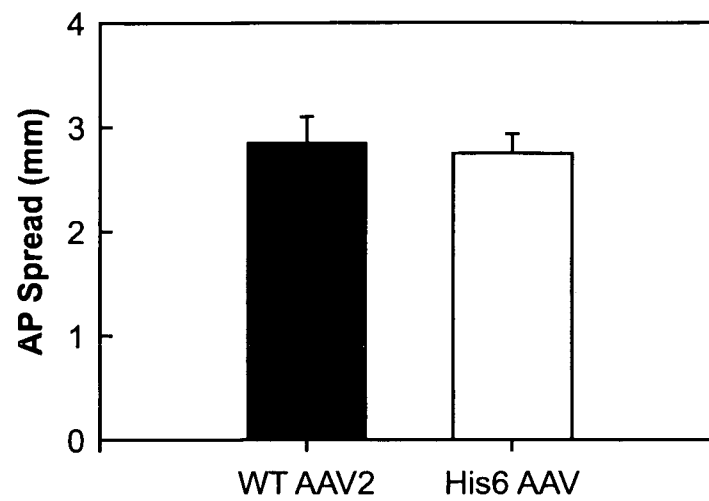


FIG. 4A

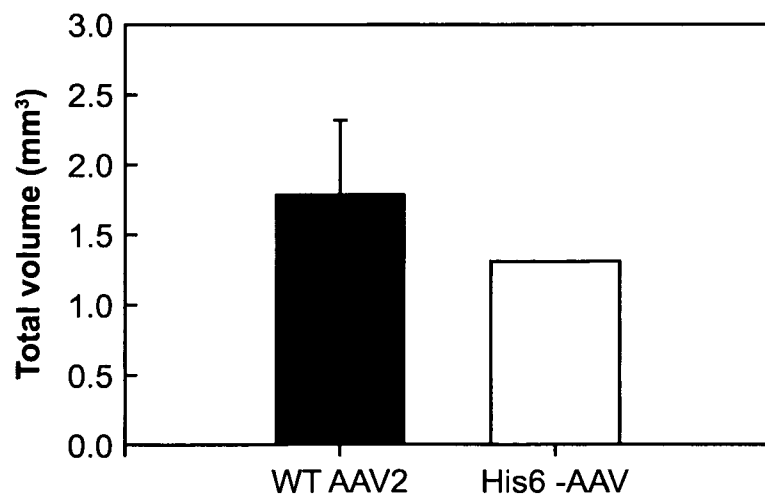


FIG. 4B

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Subtilisin prodomain

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      1              5              10              15
Ala Gly Lys Ser Asn Gly Glu Lys Lys Tyr Ile Val Gly Phe Lys

    16              20              25              30
Gln Thr Met Ser Thr Met Ser Ala Ala Lys Lys Lys Asp Val Ile

    31              35              40              45
Ser Glu Lys Gly Gly Lys Val Gln Lys Gln Phe Lys Tyr Val Asp

    46              50              55              60
Ala Ala Ser Ala Thr Leu Asn Glu Lys Ala Val Lys Glu Leu Lys

    61              65              70              75
Lys Asp Pro Ser Val Ala Tyr Val Glu Glu Asp His Val Ala His

      77
Ala Tyr (SEQ ID NO:1)

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FIG. 5A

Variant-1

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      1              5              10              15
Gly Gly Lys Ser Asn Gly Glu Lys Lys Tyr Ile Val Gly Phe Lys

    16              20              25              30
Gln Gln Phe Lys Ser Cys Ala Lys Lys Glu Asp Val Ile Ser Glu

    31              35              40              45
Lys Gly Gly Lys Leu Gln Lys Cys Phe Lys Tyr Val Asp Ala Ala

    46              50              55              60
Ser Ala Thr Leu Asn Glu Lys Ala Val Glu Glu Leu Lys Lys Asp

    61              65              70              75
Pro Ser Val Ala Tyr Val Glu Glu Asp His Leu Phe Lys Ala Leu

      77
Ala Leu (SEQ ID NO:2)

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FIG. 5B

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Variant 2

1	5	10	15
Ala Gly Lys Ser	Asn Gly Glu Lys Lys	Tyr Ile Val Gly Phe	Lys
16	20	25	30
Gln Thr Met Ser	Thr Met Ser Ala Ala	Lys Lys Lys Asp	Val Ile
31	35	40	45
Ser Glu Lys Gly	Gly Lys Val Gln Lys	Gln Phe Lys Tyr	Val Asp
46	50	55	60
Ala Ala Ser Ala	Thr Leu Asn Glu Lys	Ala Val Lys Glu	Leu Lys
61	65	70	75
Lys Asp Pro Ser	Val Ala Tyr Val Glu	Glu Asp His Val	Phe Lys
77			
Ala Met (SEQ ID NO:3)			

FIG. 5C**Variant 3**

1	5	10	15
Ala Gly Lys Ser	Asn Gly Glu Lys Lys	Tyr Ile Val Gly Phe	Lys
16	20	25	30
Gln Thr Met Ser	Thr Met Ser Ala Ala	Lys Lys Lys Asp	Val Ile
31	35	40	45
Ser Glu Lys Gly	Gly Lys Val Gln Lys	Gln Phe Lys Tyr	Val Asp
46	50	55	60
Ala Ala Ser Ala	Thr Leu Asn Glu Lys	Ala Val Lys Glu	Leu Lys
61	65	70	75
Lys Asp Pro Ser	Val Ala Tyr Val Glu	Glu Asp His Val	Phe Lys
77			
Ala Tyr (SEQ ID NO:4)			

FIG. 5D

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Variant 4

1	5	10	15
Ala Gly Lys Ser	Asn Gly Glu Lys Lys	Tyr Ile Val Gly Phe	Lys
16	20	25	30
Gln Thr Met Ser	Thr Met Ser Ala Ala	Lys Lys Lys Asp Val	Ile
31	35	40	45
Ser Glu Lys Gly	Gly Lys Val Gln Lys	Gln Phe Lys Tyr Val	Asp
46	50	55	60
Ala Ala Ser Ala	Thr Leu Asn Glu Lys	Ala Val Lys Glu Leu	Lys
61	65	70	75
Lys Asp Pro Ser	Val Ala Tyr Val Glu	Glu Asp His Val	Phe Lys
77			
Ala Phe (SEQ ID NO:5)			

FIG. 5E**Variant 5**

1	5	10	15
Ala Gly Lys Ser	Asn Gly Glu Lys Lys	Tyr Ile Val Gly Phe	Lys
16	20	25	30
Ser Gly Ile Lys	Ser Cys Ala Lys Lys	Gln Asp Val Ile Ser	Glu
31	35	40	45
Lys Gly Gly Lys	Leu Gln Lys Cys Phe	Lys Tyr Val Asp Ala	Ala
46	50	55	60
Ser Ala Thr Leu	Asn Glu Lys Ala Val	Lys Glu Leu Lys Lys	Asp
61	65	70	75
Pro Ser Val Ala	Tyr Val Glu Glu Asp	Lys Val Phe Lys	Ala Met
(SEQ ID NO:6)			

FIG. 5F

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subtilisin

1	5	10	15
Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala Pro Ala			
16	20	25	30
Leu His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val			
31	35	40	45
Ile Asp Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys Val Ala			
46	50	55	60
Gly Gly Ala Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp			
61	65	70	75
Asn Asn Ser His Gly Thr His Val Ala Gly Thr Val Ala Ala Leu			
76	80	85	90
Asn Asn Ser Ile Gly Val Leu Gly Val Ala Pro Ser Ala Ser Leu			
91	95	100	105
Tyr Ala Val Lys Val Leu Gly Ala Asp Gly Ser Gly Gln Tyr Ser			
106	110	115	120
Trp Ile Ile Asn Gly Ile Glu Trp Ala Ile Ala Asn Asn Met Asp			
121	125	130	135
Val Ile Asn Met Ser Leu Gly Gly Pro Ser Gly Ser Ala Ala Leu			
136	140	145	150
Lys Ala Ala Val Asp Lys Ala Val Ala Ser Gly Val Val Val Val			
151	155	160	165
Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly Ser Ser Ser Thr Val			
166	170	175	180
Gly Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala Val Gly Ala Val			
181	185	190	195
Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val Gly Pro Glu			
196	200	205	210
Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr Leu Pro			
211	215	220	225
Gly Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Ser Pro			
226	230	235	240
His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn			
241	245	250	255
Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Thr			
256	260	265	270
Lys Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val			
271	275		
Gln Ala Ala Ala Gln			

(SEQ ID NO:7)

FIG. 6

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Variant 1: S149

1	5	10	15
Ala Lys Cys Val Ser Tyr Gly Val Ala Gln Ile Lys Ala Pro Ala			
16	20	25	30
Leu His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val			
31	35	40	45
Leu Asp Ser Gly Ile Asp Ser Ser His Pro Asp Leu Asn Val Ala			
46	50	55	60
Gly Gly Ala Ser Phe Val Pro Ser Glu Thr Asn Pro Phe Gln Asp			
61	65	70	75
Asn Asn Ser His Gly Thr His Val Ala Gly Thr Val Leu Ala <u>Leu</u>			
76	80	85	90
<u>Asn Asn Ser Ile Gly Val Leu Gly</u> Val Ala Pro Ser Ala Ser Leu			
91	95	100	105
Tyr Ala Val Lys Val Leu Gly Ala Asp Gly Ser Gly Gln Tyr Ser			
106	110	115	120
Trp Ile Ile Asn Gly Ile Glu Trp Ala Ile Ala Asn Asn Met Asp			
121	125	130	135
Val Ile Asn Met Ser Leu Gly Gly Pro Ser Gly Ser Ala Ala Leu			
136	140	145	150
Lys Ala Ala Val Asp Lys Ala Val Ala Ser Gly Val Val Val Val			
151	155	160	165
Ala Ala Ala Gly Asn Ser Gly Thr Ser Gly Ser Ser Ser Thr Val			
166	170	175	180
Ser Tyr Pro Ala Lys Tyr Pro Ser Val Ile Ala Val Gly Ala Val			
181	185	190	195
Asp Ser Ser Asn Gln Arg Ala Pro Phe Ser Ser Val Gly Pro Glu			
196	200	205	210
Leu Asp Val Met Ala Pro Gly Val Ser Ile Cys Ser Thr Leu Pro			
211	215	220	225
Gly Gly Lys Tyr Gly Ala Leu Ser Gly Thr Ser Met Ala Ser Pro			
226	230	235	240
His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn			
241	245	250	255
Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Ala Thr			
256	260	265	270
Lys Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val			
271	275		
Glu Ala Ala Ala Gln (SEQ ID NO:8)			

FIG. 7A

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Variant 2: S160

1	5	10	15
Ala Lys Cys Val Ser Tyr Gly Val Ala Gln Ile Lys Ala Pro Ala			
16	20	25	30
Leu His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val			
31	35	40	45
Leu Asp Ser Gly Ile Asp Ser Ser His Pro Asp Leu Asn Val Ala			
46	50	55	60
Gly Gly Ala Ser Phe Val Pro Ser Glu Thr Asn Pro Phe Gln Asp			
61	65	70	75
Asn Asn Ser His Gly Thr His Val Ala Gly Thr Val Leu Ala <u>Leu</u>			
76	80	85	90
<u>Asn Asn Ser Ile Gly Val Leu Gly</u> Val Ala Pro Ser Ala Ser Leu			
91	95	100	105
Tyr Ala Val Lys Val Leu Gly Ala Asp Gly Ser Gly Gln Ala Ser			
106	110	115	120
Trp Ile Ile Asn Gly Ile Glu Trp Ala Ile Ala Asn Asn Met Asp			
121	125	130	135
Val Ile Asn Met Ser Leu Gly Ser Pro Ser Gly Ser Ala Ala Leu			
136	140	145	150
Lys Ala Ala Val Asp Lys Ala Val Ala Ser Gly Val Val Val Val			
151	155	160	165
Ala Ala Ala Gly Asn Ser Gly Thr Ser Gly Ser Ser Ser Thr Val			
166	170	175	180
Ser Tyr Pro Ala Lys Tyr Pro Ser Val Ile Ala Val Gly Ala Val			
181	185	190	195
Asp Ser Ser Asn Gln Arg Ala Pro Phe Ser Ser Val Gly Pro Glu			
196	200	205	210
Leu Asp Val Met Ala Pro Gly Val Ser Ile Cys Ser Thr Leu Pro			
211	215	220	225
Gly Gly Lys Tyr Gly Ala Leu Ser Gly Thr Ser Met Ala Ser Pro			
226	230	235	240
His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn			
241	245	250	255
Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Ala Thr			
256	260	265	270
Lys Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val			
271	275		
Glu Ala Ala Ala Gln (SEQ ID NO:9)			

FIG. 7B

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Variant 3: S189

1	5	10	15
Ala	Lys Cys Val	Ser Tyr Gly Val	Ala Gln Ile Lys Ala Pro Ala
16	20	25	30
Leu	His Ser Gln Gly Tyr Thr Gly Ser	Asn Val Lys Val Ala Val	
31	35	40	45
Leu Ala Ser Gly Ile Asp Ser Ser His	Pro Asp Leu	Asn Val Ala	
46	50	55	60
Gly Gly Ala Ser	Phe Val Pro Ser Glu Thr	Asn Pro Phe Gln Asp	
61	65	70	75
Asn Asn Ser His Gly Thr His Val Ala	Gly Thr Val	Leu Ala	<u>Leu</u>
76	80	85	90
<u>Asn Asn Ser Ile Gly Val Leu Gly</u>	Val Ala Pro Ser Ala Ser	Leu	
91	95	100	105
Tyr Ala Val Lys Val Leu Gly Ala Asp	Gly Ser Gly Gln	Ala Ser	
106	110	115	120
Trp Ile Ile Asn Gly Ile Glu Trp Ala	Ile Ala Asn Asn Met Asp		
121	125	130	135
Val Ile Asn Met Ser Leu Gly	Ser Pro Ser Gly Ser Ala Ala	Leu	
136	140	145	150
Lys Ala Ala Val Asp Lys Ala Val Ala	Ser Gly Val Val Val		
151	155	160	165
Ala Ala Ala Gly Asn	Ser Gly Thr Ser Gly Ser Ser Ser Thr	Val	
166	170	175	180
Ser Tyr Pro Ala Lys Tyr Pro Ser Val	Ile Ala Val Gly Ala	Val	
181	185	190	195
Asp Ser Ser Asn Gln Arg Ala	Pro Phe Ser Ser Val Gly Pro	Glu	
196	200	205	210
Leu Asp Val Met Ala Pro Gly Val Ser	Ile Cys Ser Thr Leu	Pro	
211	215	220	225
Gly Gly Lys Tyr Gly Ala	Leu Ser Gly Thr Ser Met Ala Ser	Pro	
226	230	235	240
His Val Ala Gly Ala Ala Ala Leu Ile	Leu Ser Lys His Pro	Asn	
241	245	250	255
Trp Thr Asn Thr Gln Val Arg Ser Ser	Leu Glu Asn Thr	Ala Thr	
256	260	265	270
Lys Leu Gly Asp Ser Phe Tyr Tyr Gly	Lys Gly Leu Ile Asn	Val	
271	275		
Glu Ala Ala Ala Gln	(SEQ ID NO:10)		

FIG. 7C

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Variant 4: S190

1	5	10	15
Ala Lys Cys Val Ser Tyr Gly Val Ala Gln Ile Lys Ala Pro Ala			
16	20	25	30
Leu His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val			
31	35	40	45
Leu Ser Ser Gly Ile Asp Ser Ser His Pro Asp Leu Asn Val Ala			
46	50	55	60
Gly Gly Ala Ser Phe Val Pro Ser Glu Thr Asn Pro Phe Gln Asp			
61	65	70	75
Asn Asn Ser His Gly Thr His Val Ala Gly Thr Val Leu Ala <u>Leu</u>			
76	80	85	90
<u>Asn Asn Ser Ile Gly Val Leu Gly</u> Val Ala Pro Ser Ala Ser Leu			
91	95	100	105
Tyr Ala Val Lys Val Leu Gly Ala Asp Gly Ser Gly Gln Ala Ser			
106	110	115	120
Trp Ile Ile Asn Gly Ile Glu Trp Ala Ile Ala Asn Asn Met Asp			
121	125	130	135
Val Ile Asn Met Ser Leu Gly Ser Pro Ser Gly Ser Ala Ala Leu			
136	140	145	150
Lys Ala Ala Val Asp Lys Ala Val Ala Ser Gly Val Val Val Val			
151	155	160	165
Ala Ala Ala Gly Asn Ser Gly Thr Ser Gly Ser Ser Ser Thr Val			
166	170	175	180
Ser Tyr Pro Ala Lys Tyr Pro Ser Val Ile Ala Val Gly Ala Val			
181	185	190	195
Asp Ser Ser Asn Gln Arg Ala Pro Phe Ser Ser Val Gly Pro Glu			
196	200	205	210
Leu Asp Val Met Ala Pro Gly Val Ser Ile Cys Ser Thr Leu Pro			
211	215	220	225
Gly Gly Lys Tyr Gly Ala Leu Ser Gly Thr Ser Met Ala Ser Pro			
226	230	235	240
His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn			
241	245	250	255
Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Ala Thr			
256	260	265	270
Lys Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val			
271	275		
Glu Ala Ala Ala Gln (SEQ ID NO:11)			

FIG. 7D

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Variant 2: S196

1	5	10	15
Ala Lys Cys Val Ser Tyr Gly Val Ala Gln Ile Lys Ala Pro Ala			
16	20	25	30
Leu His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val			
31	35	40	45
Leu Val Ser Gly Ile Asp Ser Ser His Pro Asp Leu Asn Val Ala			
46	50	55	60
Gly Gly Ala Ser Phe Val Pro Ser Glu Thr Asn Pro Phe Gln Asp			
61	65	70	75
Asn Asn Ser His Gly Thr His Val Ala Gly Thr Val Leu Ala <u>Leu</u>			
76	80	85	90
<u>Asn Asn Ser Ile Gly Val Leu Gly</u> Val Ala Pro Ser Ala Ser Leu			
91	95	100	105
Tyr Ala Val Lys Val Leu Gly Ala Asp Gly Ser Gly Gln Ala Ser			
106	110	115	120
Trp Ile Ile Asn Gly Ile Glu Trp Ala Ile Ala Asn Asn Met Asp			
121	125	130	135
Val Ile Asn Met Ser Leu Gly Ser Pro Ser Gly Ser Ala Ala Leu			
136	140	145	150
Lys Ala Ala Val Asp Lys Ala Val Ala Ser Gly Val Val Val Val			
151	155	160	165
Ala Ala Ala Gly Asn Ser Gly Thr Ser Gly Ser Ser Ser Thr Val			
166	170	175	180
Ser Tyr Pro Ala Lys Tyr Pro Ser Val Ile Ala Val Gly Ala Val			
181	185	190	195
Asp Ser Ser Asn Gln Arg Ala Pro Phe Ser Ser Val Gly Pro Glu			
196	200	205	210
Leu Asp Val Met Ala Pro Gly Val Ser Ile Cys Ser Thr Leu Pro			
211	215	220	225
Gly Gly Lys Tyr Gly Ala Leu Ser Gly Thr Ser Met Ala Ser Pro			
226	230	235	240
His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn			
241	245	250	255
Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Ala Thr			
256	260	265	270
Lys Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val			
271	275		
Glu Ala Ala Ala Gln (SEQ ID NO:12)			

FIG. 7E

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GenBank X00165

```
181   gcagggaa atcaaacggg gaaaagaaat atattgtcgg gtttaaacag acaatgagca
241   cgatgagcgc cgctaagaag aaagatgtca tttctgaaaa aggcgggaaa gtgcaaaagc
301   aattcaaata tgtagacgca gcttcagcta cattaaacga aaaagctgta aaagaattga
361   aaaaagaccc gagcgtcgct tacgttgaag aagatcacgt agcacatgcg tac (SEQ ID
NO:15)
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FIG. 9

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GenBank Accession No. YP_680426

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1 maadgylpdw ledtlsegir qwwklkpgpp ppkpaerhkd dsrglvlpgy kylgpfngld
61 kgepvneada aalehdkayd rqlsdgdnpy lkynhadaef qerlkedtsf ggnlgravfq
121 akkrvleplg lveepvktap gkkrpvehsp vepdsssgtg kagqqparkr lnfgqtgdad
181 svpdpqplgq ppaapsglgt ntmatgsgap madnnegadg vgnssgnwhc dstwmgdrvi
241 ttstrtwalp tynnhlykqi ssqsgasndn hyfgystpwg yfdfnrfhch fsprdwqrli
301 nnnwgfrpkr lnfklfniqv kevtqndgtt tiannltstv qvftdseyql pyvlgsahqg
361 clppfpadvf mvpqygyltl nngsqavgrs sfycleyfps qmlrtgnnft fsytfedvpf
421 hssyahsqs1 drlmnplidq ylyylsrtnt psgtttqsrl qfsqagasdi rdqsrnwlpg
481 pcyrqqrsvk tsadnnnsey swtgatkyhl ngrdslvnpg pamashkdde ekffpqsgvl
541 ifgkqgsekt nvdiekvmit deeeirttnp vateqygsvs tnlqrgrnqa atadvntqgv
601 lpgmvwqdrd vylqgpiwak iphtdghfhp splmggfglk hpppqilikn tpvpanpstt
661 fsaakfasfi tqystgqvsv eiewelqken skrwnpeiqy tsnynksvnnv dftvdtngvy
721 seprpigtry ltrnl (SEQ ID NO:16)
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FIG. 10

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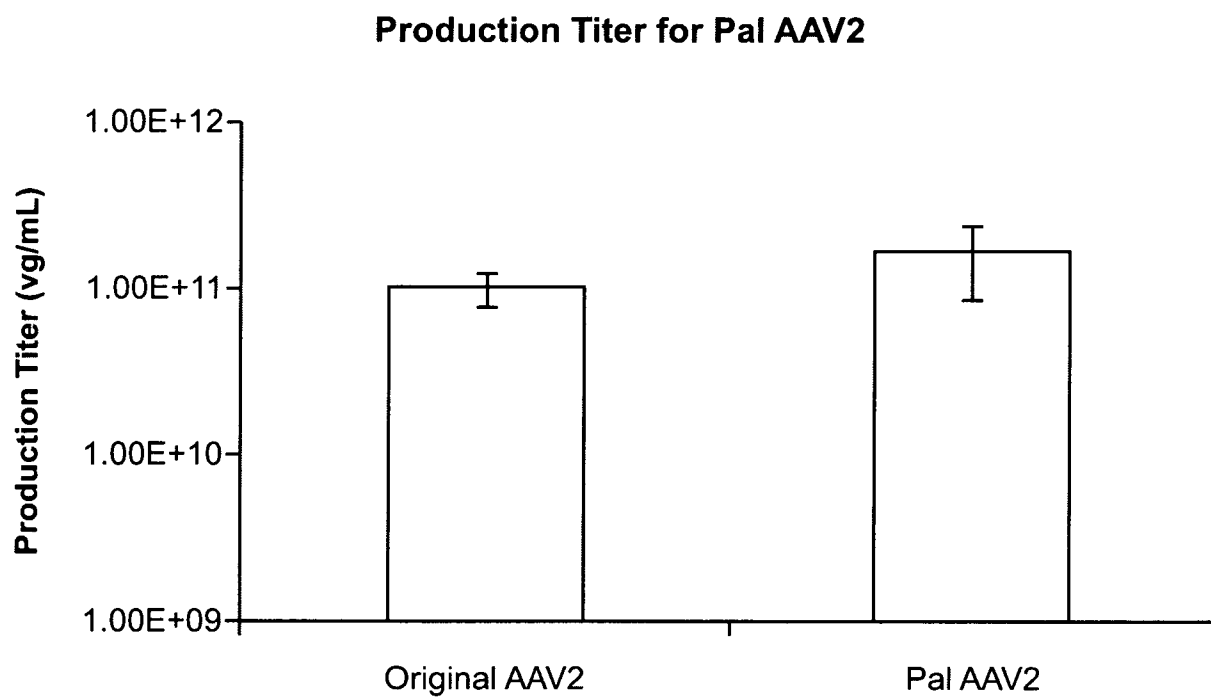


FIG. 11

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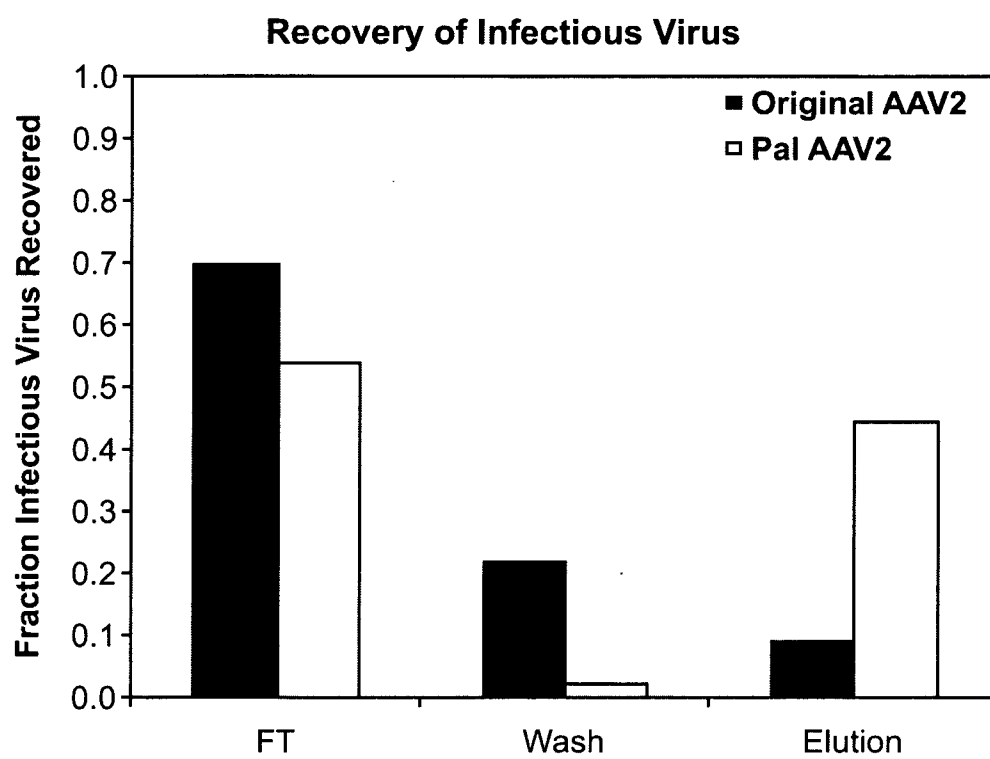


FIG. 12

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 08/04312

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 7/02 (2008.04)

USPC - 435 239; 435/803

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

USPC-435/239; 435/803

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/235.1 (text search, see terms below)

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

PubWEST (PGPB,USPT,EPAB,JPAB); Google/Scholar; PubMed (text search, see terms below)

Search Terms: Subtilisin, prodomain, metal ion, affinity, metal affinity chromatography, metal chelate affinity, adenovirus, adeno-associated virus, capsid, purification, isolation

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -----	Koerber et al. Engineering Adeno-associated virus for one-step purification via immobilized metal affinity chromatography. Human GeneTherapy, April 1, 2007, vol 18, pp 367-378, particularly Abstract, Figure 1, (page 368, paras 4,6), (page 369, paras 2-4), (page 371, para 4), (page 372, para 2), (page 374, para 5).	1-7, 12-14, 17-22, 24, 25 -----
Y		8-11, 15, 16, 23
Y	US 2006/0134740 A1 (BRYAN) 22 June 2006 (22.06.2006), particularly (para [0020]), (para [0051]), (para [0160]).	8-11, 15, 16, 23

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"&" document member of the same patent family

Date of the actual completion of the international search

30 May 2008 (30.05 2008)

Date of mailing of the international search report

27 JUN 2008

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