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TITLE OF THE INVENTION (280 characters max)					
MUTANT ADENO-ASSOCIATED VIRUS VIRIONS AND METHODS OF USE THEREOF					
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☒ Application Data Sheet. See 37 CFR 1.76					
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.					
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Respectfully submitted,

Date 06/30/2003

SIGNATURE

REGISTRATION NO. 42,344
(if appropriate)TYPED or PRINTED NAME Paula A. BordenDocket Number: BERK-024PRVTELEPHONE (650) 327-3400**USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT**

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MUTANT ADENO-ASSOCIATED VIRUS VIRIONS AND METHODS OF USE THEREOF

FIELD OF THE INVENTION

[0001] The present invention is in the field of recombinant adeno-associated virus vectors.

BACKGROUND OF THE INVENTION

[0002] Adeno-associated virus (AAV) is a 4.7 kb, single stranded DNA virus that contains two open reading frames, *rep* and *cap*. The first gene encodes four proteins necessary for genome replication (Rep78, Rep68, Rep52, and Rep40), and the second expresses three structural proteins (VP1-3) that assemble to form the viral capsid. As its name implies, AAV is dependent upon the presence of a helper virus, such as an adenovirus or herpesvirus, for active replication. In the absence of a helper it establishes a latent state in which its genome is maintained episomally or integrated into the host chromosome. To date, eight homologous primate AAV serotypes and numerous non-human primate types have been identified, although AAV2 is the best characterized as a gene delivery vehicle.

[0003] In 1989 a recombinant AAV2 (rAAV) gene delivery vector system was first generated, and vectors based on AAV have subsequently been shown to offer numerous major advantages. First, vectors based on AAV are extremely safe, since wild-type AAV is nonpathogenic and has no etiologic association with any known diseases. In addition, AAV offers the capability for highly efficient gene delivery and sustained transgene expression in numerous tissues, including muscle, lung, and brain. Furthermore, AAV has enjoyed success in human clinical trials.

[0004] Despite this success, vector design problems remain. One major concern is the fact that much of the human population has already been exposed to various AAV serotypes, and as a result a significant fraction of any future patient population harbors neutralizing antibodies (NABs) that block gene delivery. Additional problems with rAAV vectors include limited tissue dispersion for serotypes that employ heparan sulfate as a receptor (AAV2 and 3), poor infection of non-permissive cell types such as stem

cells, challenges with high efficiency targeting of gene delivery to selected cell populations, and a finite transgene carrying capacity.

[0005] There is a need in the art for improved AAV vectors that evade neutralization by serum antibodies to AAV. The present invention addresses this need.

Literature

[0006] Halbert et al. (2000) *J Virol* 74, 1524-32; Blacklow et al. (1971) *Am J Epidemiol* 94, 359-66. (1971); Erles et al. (1999) *J Med Virol* 59, 406-11; Moskalenko et al. (2000) *J Virol* 74, 1761-6; Wobus et al. (2000) *J Virol* 74, 9281-93; Sun et al. (2003) *Gene Ther* 10, 964-76; Nguyen (2001) *Neuroreport* 12, 1961-4; Davidson et al. (2000) *Proc Natl Acad Sci U S A* 97, 3428-32; Rabinowitz et al. (1999) *Virology* 265, 274-85; Opie et al. (2003) *J Virol* 77, 6995-7006.

SUMMARY OF THE INVENTION

[0007] The present invention provides mutant adeno-associated virus (AAV) that exhibit altered capsid properties, e.g., reduced binding to neutralizing antibodies in serum and/or altered heparan binding and/or altered infectivity of particular cell types. The present invention further provides libraries of mutant AAV comprising one or more mutations in a capsid gene. The present invention further provides methods of generating the mutant AAV and mutant AAV libraries, and compositions comprising the mutant AAV. The present invention further provides recombinant AAV (rAAV) virions that comprise a mutant capsid protein. The present invention further provides nucleic acids comprising nucleotide sequences that encode mutant capsid proteins, and host cells comprising the nucleic acids. The present invention further provides methods of delivering a gene product to an individual, the methods generally involving administering an effective amount of a subject rAAV virion to an individual in need thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] Figures 1a and 1b depict heparin binding characteristics of wild type AAV versus the viral library.

[0009] Figures 2a and 2b depict generation of antibody neutralization escape mutants.

[0010] Figure 3 depicts *cap* nucleotide sequence alignment of neutralizing antibody escape mutants.

DEFINITIONS

- [0011] 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.
- [0012] "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").
- [0013] 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.
- [0014] 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.
- [0015] "Packaging" refers to a series of intracellular events that result in the assembly and encapsidation of an AAV particle.
- [0016] 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".
- [0017] 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.

[0018] "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.

[0019] 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.

[0020] 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).

[0021] 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.

[0022] A "gene" refers to a polynucleotide containing at least one open reading frame that is capable of encoding a particular protein after being transcribed and translated.

[0023] "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.

[0024] 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.

[0025] "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.

[0026] 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.

[0027] "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.

[0028] "Genetic alteration" refers to a process wherein a genetic element 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.

[0029] A cell is said to be "stably" altered, transduced, or transformed with a genetic sequence if the sequence is available to perform its function during extended culture of the cell in vitro. In preferred examples, such a cell is "inheritably" altered in that a genetic alteration is introduced which is also inheritable by progeny of the altered cell.

[0030] 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, or conjugation with a labeling component. Polypeptides such as "CFTR", "p53", "E1A" and the like, when discussed in the context of gene therapy 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, E1A genes, and other such genes for use in gene therapy (typically 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.

[0031] An "isolated" plasmid, 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 preferred. Thus, for example, a 2-fold enrichment is preferred, 10-fold enrichment is more preferred, 100-fold enrichment is more preferred, 1000-fold enrichment is even more preferred.

[0032] As used herein, the terms "treatment," "treating," and the like, refer to obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse affect attributable to the disease. "Treatment," as used herein, covers any treatment of a disease in a mammal, particularly in a human, and includes: (a) preventing the disease from occurring in a subject which may be predisposed to the disease or at risk of acquiring the disease but has not yet been diagnosed as having it; (b) inhibiting the disease, i.e., arresting its development; and (c) relieving the disease, i.e., causing regression of the disease.

[0033] The terms “individual,” “host,” “subject,” and “patient” are used interchangeably herein, and refer to a mammal, including, but not limited to, primates, including simians and humans.

[0034] 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.

[0035] 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.

[0036] 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.

[0037] It must be noted that as used herein and in the appended claims, the singular forms “a”, “and”, and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “an rAAV vector” includes a plurality of such vectors and reference to “the mutant AAV capsid protein” includes reference to one or more mutant AAV capsid proteins and equivalents thereof known to those skilled in the art, and so forth.

[0038] 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 OF THE INVENTION

- [0039] The present invention provides mutant adeno-associated virus (AAV) that exhibit altered capsid properties, e.g., reduced binding to neutralizing antibodies in serum and/or altered heparan binding and/or altered infectivity of particular cell types. The present invention further provides libraries of mutant AAV comprising one or more mutations in a capsid gene. The present invention further provides methods of generating the mutant AAV and mutant AAV libraries, and compositions comprising the mutant AAV. The present invention further provides recombinant AAV (rAAV) virions that comprise a mutant capsid protein. The present invention further provides nucleic acids comprising nucleotide sequences that encode mutant capsid proteins, and host cells comprising the nucleic acids. The present invention further provides methods of delivering a gene product to an individual, the methods generally involving administering an effective amount of a subject rAAV virion to an individual in need thereof.
- [0040] A subject mutant AAV virion or a subject rAAV virion exhibits one or more of the following properties: 1) increased heparan sulfate binding affinity relative to wild-type AAV; 2) decreased heparan sulfate binding affinity relative to wild-type AAV; 3) increased infectivity of a cell that is resistant to infection with AAV; 4) increased evasion of neutralizing antibodies; and 5) increased ability to cross an endothelial cell layer.
- [0041] A subject nucleic acid encoding a mutant AAV capsid protein is useful for generating recombinant AAV virions that exhibit altered properties such as increased heparan sulfate binding, decreased heparan sulfate binding, increased infectivity of a cell that is resistant to infection with AAV, increased evasion of neutralizing antibodies, increased ability to cross an endothelial cell layer, and the like. A subject rAAV virion is useful for delivering a gene product to an individual.
- [0042] Cell membrane-associated heparan sulfate proteoglycan is a primary cell surface receptor for AAV, e.g., AAV-2. Increased heparan sulfate binding is advantageous where, e.g., the rAAV particle is being delivered in a localized manner, e.g., where

diffusion of the rAAV particle away from the site of delivery is not desired. Many cell types produce heparan sulfate, which remains on the surface of the cell or in the immediate environment of the cell. Thus, an rAAV virion with increased heparan sulfate binding affinity would remain relatively close to the site of administration. For example, localized delivery of a subject rAAV virion is advantageous for delivery of a gene product to a tumor that is localized to a particular anatomical site (but not, e.g., to surrounding non-cancerous tissue), for delivery of a gene product to a diseased cardiac vessel (but not to the surrounding healthy heart tissue), etc.

[0043] Increased heparan binding is also advantageous in that it confers increased infectivity of cell types that are typically refractory to infection with AAV, e.g., non-permissive cell types. Such cell types include those with relatively low amounts of heparan sulfate on their surface. Increased heparan sulfate binding affinity allows an increased level of binding to cells that have relatively low levels of surface heparan sulfate, and therefore leads to increased infectivity of these cells. An example of cells that are refractory to infection with AAV is a stem cell. Thus, a subject rAAV virion is advantageous because it can infect stem cells and can deliver gene products to stem cells.

[0044] Decreased heparan sulfate binding is advantageous for therapeutic strategies in which more widespread, or systemic delivery of a subject rAAV virion is desired. Such rAAV virions diffuse away from the site of administration, and thus infect a greater number of cells than rAAV virions with wild-type capsid protein(s).

[0045] Decreased binding to neutralizing antibodies is advantageous. Neutralizing antibodies bind to wild-type capsid proteins. Binding of neutralizing antibodies to wild-type capsid proteins may have several effects, including limiting the residence time of an rAAV virions that comprises wild-type capsid proteins in the viral particle, preventing the virus from binding to the cell surface, aggregating the virus, induction of structural alterations in the capsid, and prevention of viral disassembly and uncoating (a step necessary to release the DNA). An rAAV particle that has decreased binding to neutralizing antibodies thus has increased capacity to infect cells, and increased residence time in the body of an individual administered with the rAAV virion. Thus, the effective duration of delivery of gene product is increased.

[0046] Increased ability to cross an endothelial cell layer allows the rAAV virion to gain access to tissues and cells that are separated from the site of administration by an endothelial cell layer. For example, the blood-brain barrier, the tumor vasculature, and the cardiovascular system all present endothelial cell layers that form a barrier to access of a particular anatomical site. A subject rAAV virion thus may exhibit one or more of the following properties: 1) increased ability to cross the blood-brain barrier; 2) increased ability to cross the tumor vasculature and infect tumor cells; and 3) increased ability to cross the endothelial layer within the heart.

MUTANT ADENO-ASSOCIATED VIRUS VIRIONS

[0047] The present invention provides mutant adeno-associated virus comprising mutant capsid proteins that exhibit altered capsid properties. By virtue of comprising one or more mutant capsid proteins, a subject mutant AAV exhibits one or more of the following properties: 1) increased heparan binding affinity relative to wild-type AAV; 2) decreased heparan binding affinity relative to wild-type AAV; 3) increased infectivity of a cell that is resistant to infection with AAV; 4) increased evasion of neutralizing antibodies; and 5) increased ability to cross an endothelial cell layer.

[0048] In some embodiments, a capsid protein encoded by a subject mutant AAV exhibits increased binding affinity to heparan sulfate relative to wild-type AAV. In these embodiments, a capsid protein encoded by a subject mutant AAV exhibits at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, 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 2-fold, at least 2.5-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 50 fold, at least about 75-fold, or at least about 100-fold or more, higher affinity for heparan sulfate than wild-type AAV capsid. Because heparin is a molecule that is structurally similar to heparan sulfate, heparin is frequently used to determine experimentally whether a capsid protein has altered binding to heparan sulfate. For example, whereas the binding affinity of AAV2 to heparin has a $K(d)$ value of approximately 2.0 nM, a subject mutant AAV has a binding affinity to heparin that is at least about 10%, at least about 15%, at least about 20%, at least about

25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, 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 2-fold, at least 2.5-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 50 fold, at least about 75-fold, or at least about 100-fold or more, higher than the affinity of wild-type AAV2 to heparin. Typically, wild-type AAV elutes from a heparan affinity chromatography medium with a NaCl concentration in a range of from about 450 mM to about 550 mM. For example, a subject mutant AAV elutes from a heparan affinity chromatography medium with a NaCl concentration of greater than about 550 mM, e.g., from about 575 mM NaCl to about 600 mM NaCl, from about 600 mM NaCl to about 625 mM NaCl, from about 625 mM NaCl to about 650 mM NaCl, from about 650 mM NaCl to about 675 mM NaCl, from about 675 mM NaCl to about 700 mM NaCl, from about 700 mM NaCl to about 725 mM NaCl, from about 725 mM NaCl to about 750 mM NaCl, from about 750 mM NaCl to about 775 mM NaCl, or from about 775 mM NaCl to about 800 mM NaCl, or higher.

[0049] In other embodiments, a subject mutant AAV exhibits a lower affinity for heparan sulfate than wild-type AAV. In these embodiments, a subject rAAV vector, when packaged in a viral particle, has at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, or at least about 85% lower affinity for heparan than wild-type AAV. For example, a subject rAAV vector, when packaged into a viral particle, elutes from a heparan affinity chromatography medium with concentration of NaCl in the range of from about 440 mM NaCl to about 400 mM NaCl, from about 400 mM NaCl to about 375 mM NaCl, from about 375 mM NaCl to about 350 mM NaCl, from about 350 mM NaCl to about 325 mM NaCl, from about 325 mM NaCl to about 300 mM NaCl, from about 300 mM NaCl to about 275 mM NaCl, from about 275 mM NaCl to about 250 mM NaCl, from about 250 mM NaCl to about 225 mM NaCl, from about 225 mM NaCl to about 200 mM NaCl or lower.

[0050] Heparan binding affinity can be determined using any known assay. For example, affinity of variant capsids for heparan sulfate can be measured by binding viral particles to immobilized heparin. See, e.g., Qui et al. (2000) *Virology* 269:137-147.

[0051] In some embodiments, a subject mutant AAV exhibits increased resistance to neutralizing antibodies compared to wild-type AAV ("wt AAV") or AAV comprising a wild-type capsid protein. In these embodiments, a subject rAAV vector has from about 10-fold to about 10,000-fold greater resistance to neutralizing antibodies than wt AAV, e.g., a subject rAAV vector 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.

[0052] In some embodiments, a subject mutant AAV exhibits decreased binding to a neutralizing antibody that binds a wild-type AAV capsid protein. For example, a subject mutant AAV 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 AAV 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.

[0053] In some embodiments, a subject mutant AAV exhibits increased *in vivo* residence time compared to a wild-type AAV. For example, a subject mutant AAV exhibits a residence time that is at least about 10%, at least about 25%, at least about 50%, at least about 100%, at least about 3-fold, at least about 5-fold, at least about 10-fold, at least about 25-fold, at least about 50-fold, at least about 100-fold, or more, longer than the residence time of a wild-type AAV.

[0054] Whether a given mutant AAV 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 AAV is contacted with a permissive cell type, e.g., 293 cells, in the presence of neutralizing antibody. A control sample contains the cells, mutant AAV, and no neutralizing antibody. After a suitable time, the cells are contacted with adenovirus, and AAV particles are detected. The level of AAV particles is compared to the amount of AAV particles that are generated in the absence of neutralizing antibody.

[0055] In some embodiments, a subject mutant AAV 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%, or more, greater infectivity of a non-permissive cell than a wild-type AAV.

[0056] Examples of cells that are relatively refractory to AAV infection include stem cells. 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.

[0057] 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.

[0058] 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; etc. Other hematopoietic "progenitor" cells of interest include cells dedicated to lymphoid lineages, e.g. immature T cell and B cell populations.

[0059] A subject mutant AAV virion comprises a mutation in at least one capsid protein (e.g., at least one of VP1, VP2, and VP3). Thus, at least one of VP1, VP2, and VP3 has at least one amino acid substitution compared to wild-type AAV capsid protein. In some embodiments, at least one of VP1, VP2, and VP3 has from one to about 25 amino acid substitutions compared to wild-type AAV VP1, VP2, and VP3, e.g., from about one to about 5, from about 5 to about 10, from about 10 to about 15, from about 15 to about 20, or from about 20 to about 25 amino acid substitutions compared to wild-type AAV VP1, VP2, and VP3. Alternatively, a subject mutant AAV virion comprises one or more amino acid deletions and/or insertions in at least one capsid protein relative to wild-type capsid protein. In some embodiments, a subject mutant AAV virion comprises one or more amino acid substitutions and/or deletions and/or insertions in a capsid protein relative to a wild-type capsid protein.

[0060] In some embodiments, a subject mutant AAV virion comprises wild-type Rep78, Rep68, Rep52, and Rep40 proteins. In other embodiments, a subject mutant AAV comprises, in addition to one or more mutant capsid proteins, one or more mutations in one or more of Rep78, Rep68, Rep52, and Rep40 proteins.

NUCLEIC ACIDS AND HOST CELLS

[0061] The present invention provides nucleic acids comprising nucleotide sequences encoding a mutant AAV capsid protein, as well as host cells comprising a subject nucleic acid. The nucleic acids and host cells are useful for generating rAAV virions, as described below. A subject nucleic acid encodes one or more of VP1, VP2, and VP3 comprising one or more amino acid substitutions. A subject nucleic acid comprises a nucleotide sequence encoding at least one of VP1, VP2, and VP3, wherein the encoded capsid protein comprises from one to about 15 amino acid substitutions compared to a wild-type AAV capsid protein, e.g., from about one to about 5, from about 5 to about 10, from about 10 to about 15, from about 15 to about 20, or from about 20 to about 25 amino acid substitutions compared to a wild-type AAV capsid protein. The encoded capsid protein may, alternatively or in addition, comprise one or more amino acid deletions and/or insertions relative to a wild-type AAV capsid protein.

[0062] The present invention further provides host cells, e.g., isolated host cells, comprising a subject nucleic acid. A subject host cell is typically an isolated cell, e.g., a cell in *in vitro* culture. A subject host cell is useful for producing a subject rAAV virion, as described below. Where a subject host cell is used to produce a subject rAAV 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.

[0063] 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.

[0064] 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 cell lines include, but are not limited to, 293 cells, COS cells, HeLa cells, Vero cells, 3T3 mouse fibroblasts, C3H10T1/2 fibroblasts, CHO cells, and the like.

[0065] 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, an rAAV virion is generated using a subject host cell.

rAAV VIRIONS

[0066] A mutant capsid protein may be incorporated into an AAV 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 recombinant AAV virion that comprises a mutant capsid protein has ("rAAV virions"). Thus, the present invention provides rAAV virions that comprise a mutant capsid protein, as described above; and a heterologous nucleic acid. A subject rAAV virion is useful for introducing a gene product into an individual.

[0067] A subject rAAV virion comprises a mutant capsid protein, as described above. By virtue of comprising a mutant capsid protein, a subject rAAV virion exhibits one or more of the following properties: 1) increased heparan binding affinity relative to wild-type AAV; 2) decreased heparan binding affinity relative to wild-type AAV; 3) increased infectivity of a cell that is resistant to infection with AAV; 4) increased evasion of neutralizing antibodies; and 5) increased ability to cross an endothelial cell layer.

[0068] In some embodiments, a subject rAAV virion exhibits increased binding affinity to heparan relative to a wild-type AAV virion. In these embodiments, a capsid protein encoded by a subject mutant AAV exhibits at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, 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 2-fold, at least

2.5-fold, at least about 5-fold, at least about 10-fold, at least about 15-fold, at least about 50 fold, at least about 75-fold, or at least about 100-fold or more, higher affinity for heparan than wild-type AAV capsid. Typically, wild-type AAV elutes from a heparan affinity chromatography medium with a NaCl concentration in a range of from about 450 mM to about 550 mM. For example, a subject rAAV virion elutes from a heparan affinity chromatography medium with a NaCl concentration of greater than about 550 mM, e.g., from about 575 mM NaCl to about 600 mM NaCl, from about 600 mM NaCl to about 625 mM NaCl, from about 625 mM NaCl to about 650 mM NaCl, from about 650 mM NaCl to about 675 mM NaCl, from about 675 mM NaCl to about 700 mM NaCl, from about 700 mM NaCl to about 725 mM NaCl, from about 725 mM NaCl to about 750 mM NaCl, from about 750 mM NaCl to about 775 mM NaCl, or from about 775 mM NaCl to about 800 mM NaCl, or higher.

[0069] In other embodiments, a subject rAAV virion exhibits a lower affinity for heparan than wild-type AAV. In these embodiments, a subject rAAV virion has at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, or at least about 85% lower affinity for heparan than wild-type AAV. For example, a subject rAAV virion elutes from a heparan affinity chromatography medium with concentration of NaCl in the range of from about 440 mM NaCl to about 400 mM NaCl, from about 400 mM NaCl to about 375 mM NaCl, from about 375 mM NaCl to about 350 mM NaCl, from about 350 mM NaCl to about 325 mM NaCl, from about 325 mM NaCl to about 300 mM NaCl, from about 300 mM NaCl to about 275 mM NaCl, from about 275 mM NaCl to about 250 mM NaCl, from about 250 mM NaCl to about 225 mM NaCl, from about 225 mM NaCl to about 200 mM NaCl or lower.

[0070] Heparan binding affinity can be determined using any known assay. For example, affinity of variant capsids for heparan sulfate can be measured by binding viral particles to immobilized heparin. See, e.g., Qui et al. (2000) *Virology* 269:137-147.

[0071] 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.

[0072] 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.

[0073] 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.

[0074] 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.

[0075] 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%, or more, greater infectivity of a non-permissive cell than a wild-type AAV or an rAAV virion comprising wild-type capsid protein.

[0076] Examples of cells that are relatively refractory to AAV infection include stem cells. 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.

[0077] 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.

[0078] 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; etc. Other hematopoietic "progenitor" cells of interest include cells dedicated to lymphoid lineages, e.g. immature T cell and B cell populations.

[0079] 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.

[0080] Whether a given rAAV virion exhibits an increased ability to cross an endothelial cell layer can be determined experimentally using well-known systems.

[0081] A subject rAAV virion comprises a mutation in at least one capsid protein (e.g., at least one of VP1, VP2, and VP3). Thus, at least one of VP1, VP2, and VP3 has at least one amino acid substitution compared to wild-type AAV capsid protein. In some embodiments, at least one of VP1, VP2, and VP3 has from one to about 25 amino acid substitutions compared to wild-type AAV VP1, VP2, and VP3, e.g., from about one to about 5, from about 5 to about 10, from about 10 to about 15, from about 15 to about 20, or from about 20 to about 25 amino acid substitutions compared to wild-type AAV VP1,

VP2, and VP3. Alternatively, a subject rAAV virion comprises one or more amino acid deletions and/or insertions in at least one capsid protein relative to wild-type capsid protein. In some embodiments, a subject rAAV virion comprises one or more amino acid substitutions and/or deletions and/or insertions in a capsid protein relative to a wild-type capsid protein.

Generation of subject rAAV virions

[0082] 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.

[0083] 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.

[0084] 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.

1. rAAV vector

[0085] A subject rAAV virion, including the heterologous DNA of interest, can be produced using standard methodology, known to those of skill in the art. The methods generally involve the steps of (1) introducing an 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.

[0086] 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.

[0087] 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.

[0088] Suitable DNA molecules for use in AAV vectors will be less than about 5 kilobases (kb) in size and will include, for example, a gene that encodes a protein that is defective or missing from a recipient subject or a gene that encodes a protein having a desired biological or therapeutic effect (e.g., an antibacterial, antiviral or antitumor function).

[0089] Suitable DNA molecules include, but are not limited to, those encoding for 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 AIDS, cancer, hypercholestermia, 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.

[0090] The selected heterologous nucleotide sequence, such as EPO or another gene 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 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.).

[0091] The AAV expression vector which harbors the DNA molecule of interest 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.

[0092] 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.

[0093] 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.

[0094] 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).

[0095] 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

[0096] 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.

[0097] 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).

[0098] AAV cap proteins include VP1, VP2, and VP3, wherein at least one of VP1, VP2, and VP3 comprises at least one mutation, as described above.

[0099] 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.

[00100] 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 phosphotranferase (APH)) that allows selection in mammalian cells by conferring resistance to G418 (available from Sigma, St. Louis, Mo.). Other suitable markers are known to those of skill in the art.

3. AAV Accessory Functions

[00101] 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.

[00102] 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.

[00103] 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.

[00104] 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.

[00105] 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) 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.

[00106] Following recombinant AAV replication, rAAV virions can be purified from the host cell using a variety of conventional purification methods, such as CsCl gradients. Further, if infection is employed to express the accessory functions, residual helper virus can be inactivated, using known methods. For example, adenovirus can be inactivated by heating to temperatures of approximately 60.degree. C. for, e.g., 20 minutes or more. This treatment effectively inactivates only the helper virus since AAV is extremely heat stable while the helper adenovirus is heat labile.

[00107] The resulting rAAV virions are then ready for use for DNA delivery, such as in gene therapy applications, for the production of transgenic animals, or in vaccination.

DELIVERY OF A GENE PRODUCT

[00108] The present invention further provides methods of delivering a gene product to an individual in need thereof. The methods generally involve introducing a subject rAAV virion into an individual.

[00109] Generally, rAAV virions are introduced into a cell using either *in vivo* or *in vitro* transduction techniques. If transduced *in vitro*, the desired recipient cell will be removed from the subject, transduced with rAAV virions and reintroduced into the subject. Alternatively, syngeneic or xenogeneic cells can be used where those cells will not generate an inappropriate immune response in the subject.

[00110] Suitable methods for the delivery and introduction of transduced cells into a subject have been described. For example, cells can be transduced *in vitro* by combining recombinant AAV virions with cells e.g., in appropriate media, and screening for those cells harboring the DNA of interest using conventional techniques such as Southern blots and/or PCR, or by using selectable markers. Transduced cells can then be formulated into pharmaceutical compositions, described more fully below, and the composition introduced into the subject by various techniques, such as by intramuscular, intravenous, subcutaneous and intraperitoneal injection.

[00111] For *in vivo* delivery, the rAAV virions will be formulated into pharmaceutical compositions and will generally be administered parenterally.

[00112] Pharmaceutical compositions will comprise sufficient genetic material to produce a therapeutically effective amount of the protein of interest, i.e., an amount sufficient to reduce or ameliorate symptoms of the disease state in question or an amount sufficient to confer the desired benefit. The pharmaceutical compositions will also contain a pharmaceutically acceptable excipient. Such excipients include any pharmaceutical agent that does not itself induce the production of antibodies harmful to the individual receiving the composition, and which may be administered without undue toxicity. Pharmaceutically acceptable excipients include, but are not limited to, liquids such as water, saline, glycerol and ethanol. Pharmaceutically acceptable salts can be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides,

phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. A wide variety of pharmaceutically acceptable excipients are known in the art and need not be discussed in detail herein. Pharmaceutically acceptable excipients have been amply described in a variety of publications, including, for example, A. Gennaro (2000) "Remington: The Science and Practice of Pharmacy," 20th edition, Lippincott, Williams, & Wilkins; Pharmaceutical Dosage Forms and Drug Delivery Systems (1999) H.C. Ansel et al., eds., 7th ed., Lippincott, Williams, & Wilkins; and Handbook of Pharmaceutical Excipients (2000) A.H. Kibbe et al., eds., 3rd ed. Amer. Pharmaceutical Assoc.

[00113] Appropriate doses will depend on the mammal being treated (e.g., human or nonhuman primate or other mammal), age and general condition of the subject to be treated, the severity of the condition being treated, the particular therapeutic protein in question, its mode of administration, among other factors. An appropriate effective amount can be readily determined by one of skill in the art.

[00114] Thus, a "therapeutically effective amount" will fall in a relatively broad range that can be determined through clinical trials. For example, for *in vivo* injection, i.e., injection directly to skeletal or cardiac muscle, a therapeutically effective dose will be on the order of from about 10^6 to about 10^{15} of the rAAV virions, e.g., from about 10^8 to 10^{12} rAAV virions. For *in vitro* transduction, an effective amount of rAAV virions to be delivered to cells will be on the order of from about 10^8 to about 10^{13} of the rAAV virions. Other effective dosages can be readily established by one of ordinary skill in the art through routine trials establishing dose response curves.

[00115] Dosage treatment may be a single dose schedule or a multiple dose schedule. Moreover, the subject may be administered as many doses as appropriate. One of skill in the art can readily determine an appropriate number of doses.

[00116] In some embodiments, the present invention provides methods of delivering a gene product to a stem cell. In these embodiments, a subject rAAV virion is introduced into a stem cell, either *in vitro* or *in vivo*. 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 or neural

progenitor cells; embryonic stem cells; mesenchymal stem cells; mesodermal stem cells; etc. Other hematopoietic "progenitor" cells of interest include cells dedicated to lymphoid lineages, e.g. immature T cell and B cell populations.

[00117] Purified populations of stem or progenitor cells may be used. For example, human hematopoietic stem cells may be positively selected using antibodies specific for CD34, thy-1; or negatively selected using lineage specific markers which may include glycophorin A, CD3, CD24, CD16, CD14, CD38, CD45RA, CD36, CD2, CD19, CD56, CD66a, and CD66b; T cell specific markers, tumor specific markers, etc. Markers useful for the separation of mesodermal stem cells include FcγRII, FcγRIII, Thy-1, CD44, VLA-4α, LFA-1β, HSA, ICAM-1, CD45, Aa4.1, Sca-1, etc. Neural crest stem cells may be positively selected with antibodies specific for low-affinity nerve growth factor receptor (LNGFR), and negatively selected for the markers sulfatide, glial fibrillary acidic protein (GFAP), myelin protein P₀, peripherin and neurofilament. Human mesenchymal stem cells may be positively separated using the markers SH2, SH3 and SH4.

[00118] The cells of interest are typically mammalian, where the term refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, laboratory, sports, or pet animals, such as dogs, horses, cats, cows, mice, rats, rabbits, etc. In some embodiments, the stem cell is a human stem cell.

[00119] The cells which are employed may be fresh, frozen, or have been subject to prior culture. They may be fetal, neonate, adult. Hematopoietic cells may be obtained from fetal liver, bone marrow, blood, particularly G-CSF or GM-CSF mobilized peripheral blood, or any other conventional source. The manner in which the stem cells are separated from other cells of the hematopoietic or other lineage is not critical to this invention. As described above, a substantially homogeneous population of stem or progenitor cells may be obtained by selective isolation of cells free of markers associated with differentiated cells, while displaying epitopic characteristics associated with the stem cells.

METHODS OF GENERATING MUTANT AAV VIRIONS

[00120] The present invention provides a method of generating a mutant AAV virion comprising one or more mutations in one or more of VP1, VP2, and VP3. The method

generally involves generating a mutant AAV library; and selecting the library for capsid mutants with altered capsid properties.

[00121] In some embodiments, a given selection step is repeated two, three, four, or more times to enrich a subject AAV library for altered capsid properties. In some embodiments, following selection of an AAV library, individual clones are isolated and sequenced.

Generation of mutant AAV library

[00122] A mutant AAV library is generated that comprises one or more mutations in an AAV *cap* gene. Mutations in the AAV *cap* gene are generated using any known method. Suitable methods for mutagenesis of an AAV *cap* gene include, but are not limited to, a polymerase chain reaction (PCR)-based method, oligonucleotide-directed mutagenesis, and the like. Methods for generating mutations are well described in the art. See, e.g., Zhao et al. (1998) *Nat. Biotechnol.* 16:234-235; U.S. Patent No. 6,579,678; U.S. Patent No. 6,573,098; and U.S. Patent No. 6,582,914.

[00123] In some embodiments, a mutant AAV library comprising mutations in the *cap* gene will be generated using a staggered extension process. The staggered extension process involves amplification of the *cap* gene using a PCR-based method. The template *cap* gene is primed using specific PCR primers, followed by repeated cycles of denaturation and very short annealing/polymerase-catalyzed extension. In each cycle, the growing fragments anneal to different templates based on sequence complementarity and extend further. The cycles of denaturation, annealing, and extension are repeated until full-length sequences form. The resulting full-length sequences include at least one mutation in the *cap* gene compared to a wild-type AAV *cap* gene.

[00124] The PCR products comprising AAV *cap* sequences that include one or more mutations are inserted into a plasmid containing a wild-type AAV genome. The result is a library of AAV *cap* mutants. Thus, the present invention provides a mutant AAV *cap* gene library comprising from about 10 to about 10^{10} members, and comprising mutations in the AAV *cap* gene. A given member of the library has from about one to about 50 mutations in the AAV *cap* gene. A subject library comprises from 10 to about 10^9 distinct members, each having a different mutation(s) in the AAV *cap* gene.

[00125] Once a *cap* mutant library is generated, viral particles are produced that can then be selected on the basis of altered capsid properties. Library plasmid DNA is transfected

into a suitable host cell (e.g., 293 cells), followed by introduction into the cell of helper virus. Viral particles produced by the transfected host cells ("AAV library particles") are collected.

Library selection

[00126] Once a library is generated, it is selected for a particular capsid property. Viral particles are generated as discussed above, and subjected to one or more selection steps. Capsid properties that are selected for include, but are not limited to: 1) increased heparan binding affinity relative to wild-type AAV; 2) decreased heparan binding affinity relative to wild-type AAV; 3) increased infectivity of a cell that is resistant to infection with AAV; 4) increased evasion of neutralizing antibodies; and 5) increased ability to cross an endothelial cell layer.

1. Selection for altered heparan binding

[00127] In some embodiments, a subject library is selected for altered heparan binding, including increased heparan binding and decreased heparan binding relative to wild-type AAV virion heparan binding. AAV library particles are contacted with a heparin affinity matrix. For example, AAV library particles are loaded onto a heparin affinity column under conditions that permit binding of the AAV library particles to the heparin. Exemplary conditions include equilibration of the column with 0.15 M NaCl and 50 mM Tris at pH 7.5. After allowing the AAV library particle to bind to the heparin affinity matrix, the AAV library particle/heparin affinity matrix complex is washed with volumes of buffer containing progressively increasing concentrations of NaCl, and at each NaCl concentration, eluted AAV library particles are collected. For example, after binding the AAV library particle/heparin affinity matrix complex is washed with a volume of 50 mM Tris buffer, pH 7.5, containing 200 mM NaCl, and eluted AAV library particles are collected. The elution step is repeated with a 50 mM Tris buffer, pH 7.5, containing about 250 mM NaCl, about 300 mM NaCl, about 350 mM, about 400 mM NaCl, about 450 mM NaCl, about 500 mM NaCl, about 550 mM NaCl, about 600 mM NaCl, about 650 mM NaCl, about 700 mM NaCl, or about 750 mM NaCl.

[00128] AAV library particles that elute at NaCl concentrations lower than about 450 mM NaCl exhibit decreased heparan binding properties relative to wild-type AAV. AAV library particles that elute at NaCl concentrations higher than about 550 mM NaCl exhibit increased heparan binding properties relative to wild-type AAV.

[00129] In some embodiments, eluted AAV library particles are amplified by co-infection of permissive cells with a helper virus, and are re-fractionated on heparin affinity matrix. This step can be repeated a number of times to enrich for AAV library particles with altered heparan binding properties.

2. Selection for reduced binding to neutralizing antibodies

[00130] In some embodiments, a subject AAV library is selected for reducing binding to neutralizing antibodies that bind to and neutralize wild-type AAV virions, compared to the binding of such antibodies to wild-type AAV virions and neutralization of wild-type AAV virions. AAV library particles are contacted with neutralizing antibodies and the ability of the AAV library particles to infect a permissive host cell is tested. Typically, AAV library particles are contacted with various concentrations of neutralizing antibodies. The higher the concentration of neutralizing antibodies that is required to reduce infectivity of the AAV library particles, the more resistant the AAV particles are to neutralization.

3. Selection for increased infectivity of non-permissive cells

[00131] In some embodiments, a subject AAV library is selected for increased infectivity of non-permissive cells. AAV library particles are contacted with a non-permissive cell (e.g., a population of non-permissive cells). After a suitable amount of time to allow for infection of the cells with AAV library particles, helper virus is added, and AAV library particles that successfully infected the non-permissive cell(s) are harvested. In some embodiments, the cycle of infection, addition of helper virus, and harvesting of AAV particles is repeated one, two, three, or more times.

[00132] In the present methods, one or more selection steps may follow generation of AAV library particles. For example, in some embodiments, the method comprises selecting for increased heparan binding, followed by selecting for decreased binding to neutralizing antibodies. In other embodiments, the method comprises selecting for decreased binding to neutralizing antibodies, followed by selecting for increased heparan binding. In other embodiments, the method comprises selecting for decreased heparan binding, followed by selecting for decreased binding to neutralizing antibodies. In other embodiments, the method comprises selecting for decreased binding to neutralizing antibodies, followed by selecting for decreased heparan binding. In other embodiments,

the method comprises selecting for decreased binding to neutralizing antibodies, followed by selecting for increased infectivity of a stem cell.

EXAMPLES

[00133] 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 Centigrade, 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, second(s); min, minute(s); hr, hour(s); and the like.

Example 1:

METHODS

Library Generation and Vector Packaging

[00134] An AAV2 *cap* ORF genetic library was generated using the staggered extension process described by Zhao et al. ((1998) *Nat. Biotechnol.* 16:258-261), and the resulting *cap* product was inserted into a plasmid containing the wild type AAV2 genome. The result was transformed into *E. coli* for large scale plasmid production and purification. AAV was then produced and purified by CsCl centrifugation essentially as previously described (Kaspar et al. (2002) *Proc. Natl. Acad. Sci. USA* 99:2320-2325; and Lai et al. (2003) *Nat. Neurosci.* 6:21-27). Briefly, the library plasmid DNA was transfected into 293 human embryonic kidney cells (ATCC) using the calcium phosphate method, followed by addition of serotype 5 adenovirus (Ad5) at an MOI of 3. Virus was purified using CsCl density centrifugation. For all experiments, the AAV genomic titer was determined by extracting vector DNA as previously described (Kaspar et al. *supra*; and

Lai et al., supra) followed by quantification using real time PCR with SYBR-Green dye (Molecular Probes) and a Biorad iCycler.

Heparin Column Chromatography

[00135] Approximately 10^{12} AAV library particles were loaded onto a 1 mL HiTrap heparin column (Amersham) previously equilibrated with 0.15 M NaCl and 50 mM Tris at pH 7.5. Washes were performed using 0.75 ml volumes of the same buffer with increasing increments of 50 mM NaCl up to 750 mM, followed by a 1 M wash. As a control, rAAV-GFP was also subjected to this chromatography. To isolate individual viral clones from library fractions that eluted at different salt concentrations, viral DNA was extracted from the fractions, amplified by PCR, and inserted into a rAAV packaging plasmid based on pAd8⁷. rAAV-GFP was then packaged as described previously¹⁴⁻¹⁶ in order to analyze the ability of these mutant capsids to package rAAV vector. Capsid variants were sequenced at the U.C. Berkeley DNA sequencing facility. Finally, the affinity of the variant capsids for heparin was quantified by using the method of Qiu et al. ((2000) *Virology* 269:137-147), except that virus bound to the immobilized heparin was quantified by real time PCR.

Antisera Generation and Antibody Neutralization Screen

[00136] Polyclonal sera containing neutralizing antibodies (NABs) against AAV2 were generated in two New Zealand White rabbits in accordance with the U.C. Berkeley Animal Care and Use Committee and NIH standards for laboratory animal care. Briefly, 5×10^{10} CsCl-purified rAAV2 particles were mixed with 0.5 mL TitreMax adjuvant (CytRx) and injected into the anterior hindlimb muscle. Two boosts were performed at 3-week intervals using the same AAV dosage, followed by antiserum collection.

[00137] Both wild type and a mutant AAV library were incubated with varying amounts of serum (0-6.25 μ l) in 75 μ l of PBS (pH 7.4) for 30 minutes at room temperature, followed by addition to 2.5×10^5 293 cells in a 6-well format. After 48 hours, AAV was rescued from infected cells by addition of Ad5, and cells were harvested 24 hours later.

[00138] Individual viral clones from the library fraction that successfully infected cells even in the presence of NAB were inserted into the rAAV packaging plasmid, and rAAV-GFP was produced as above. rAAV with mutant capsids were then incubated in 5 μ l polyclonal sera as above, followed by addition to 1×10^5 293 cells. At 72 hours

post-infection, the fraction of green cells was quantified by flow cytometry at the U.C. Berkeley Cancer Center (Beckman-Coulter EPICS).

RESULTS

Library Generation

[00139] The staggered extension process (Zhao et al., supra), a PCR-based method that generates diverse genetic libraries in a manner similar to that of DNA shuffling³⁶, was used to generate a library of *cap* mutants with point mutations randomly distributed throughout VP1-3. This product was inserted into a plasmid containing the complete wild type AAV genome to yield a viral library with approximately 10⁶ independent clones, as determined by quantifying the number of colonies following bacterial transformation. To assess its degree of sequence diversity, the plasmid library was sequenced. The plasmid was then packaged into virus by transient transfection into 293 cells followed by Ad5 infection to yield a viral particle library. This large AAV library is selected for viral variants with any variety of new properties or functions, and the capsid structure conferring these new functions are readily recovered by DNA sequence analysis of the AAV genome encapsidated in the particles.

Heparin Binding Mutants

[00140] As an initial gauge of how the library's sequence diversity translated into capsid functional diversity, we subjected CsCl-purified library particles to heparin affinity chromatography with steps of increasing NaCl concentration. As previously reported (Zolotukhin et al. (1999) *Gene Ther.* 6:973-985) wild type AAV elutes from heparin between 450 and 550 mM NaCl (Figure 1). By stark contrast, the AAV mutant library elutes at a wide range of salt concentrations, from the 150 mM load to the final 750 mM fraction. This result demonstrates that the library encompasses significant sequence and functional diversity. Since its affinity for heparan sulfate limits the spread of rAAV2 vectors upon injection in vivo, lower affinity mutants may be desirable as gene delivery vectors when wide dissemination through a large tissue or region is needed. In contrast, a higher affinity mutant may be advantageous for regionally pinpointed, high level gene expression.

[00141] **Figures 1a and 1b.** Heparin binding characteristics of wild type AAV vs. the viral library. a) The heparin affinity column chromatogram of elution of wild type AAV

(blue) and the mutant library (yellow) is shown. Virus gradually elutes from the column as the NaCl concentration is increased. b) Chromatograms of pools from the mutant library selected for lower (blue) and higher (yellow) heparin affinities.

[00142] To isolate mutants with both low and high heparin affinity, the 150 mM and 700 mM NaCl fractions from the initial library were separately amplified by co-infection of 293 cells with Ad5, and refractionated on the heparin column. After three rounds of enrichment, the majority of the two resulting viral pools eluted from the column at 150 mM and 750 mM (Figure 1b). Importantly, since each round of enrichment involved 293 cell infection, these pools are still composed of highly infectious virus. Next, individual capsid clones were isolated from each of these salt fractions and sequenced.

[00143] The fact that these mutants eluted from the heparin column at different salt concentrations indicates that they had different affinities for heparin. To accurately measure these affinities, however, we adapted the method of Qiu et al supra. By performing Scatchard binding analysis of virus to heparin immobilized to microtiter plates, we determined that the affinity of the mutants varied from wild-type AAV.

Neutralizing Antiserum Escape Mutants

[00144] The isolation of functionally diverse heparin binding mutants from our AAV mutant library demonstrates the utility of this approach for creating vectors with novel properties. We next proceeded to apply this approach to a much more significant problem: vector elimination by neutralizing antibodies. Rabbit anti-AAV2 neutralizing antiserum was generated, and a 1:1500 serum dilution was sufficient to inhibit rAAV-GFP gene delivery to 293 cells by 50%, comparable to NAB titers in human serum.

[00145] **Figures 2a and 2b.** Generation of antibody neutralization escape mutants. a) Virus was incubated with antiserum before addition to 293 cells. The level of virus that successfully evaded antibody neutralization as compared to control infection in the absence of antiserum was measured by addition of Ad5 and titring of rescued AAV by quantitative PCR on AAV genomes. b) The data on individual variants is presented.

[00146] We next isolated antibody escape mutants from the viral library. During successive rounds of selection, the library was subjected to increasing stringencies, where stringency is defined as the ratio of the dilution necessary to reduce rAAV2 with wild type capsid by 50% (a 1500-fold for the rabbit antiserum) to the dilution actually

used. Therefore, the more antiserum added to the virus, the higher the stringency. After three rounds of selection, followed by co-infection of 293 cells with Ad5 for amplification, the resulting viral pool was less susceptible to antibody neutralization as compared to wild type virus. We next analyzed five individual capsid clones from this pool by using them to package rAAV-GFP. Figure 4 demonstrates that the resulting recombinant vector is between 100 and 1000-fold more resistant to neutralizing antibodies. This is a therapeutically relevant result.

[00147] Neutralizing antibody inhibition of rAAV gene delivery. a) rAAV2 with wild type capsid was added to 293 cells and visualized after 24 hours. Virus is shown in green (Alexa 488), microtubules in red (Alexa 546), and the nucleus in blue (TO-PRO-3). b) After preincubation in neutralizing antiserum, the intracellular transport of rAAV2 with wild type capsid is significantly inhibited. c) An antibody evasion rAAV2 variant has a similar intracellular distribution to that of rAAV2. d) Furthermore, cellular delivery of this mutant is not as significantly altered in the presence of the antiserum.

[00148] In **Figure 3**, the *cap* nucleotide sequences of two neutralizing antibody escape mutants is shown, compared to wild-type *cap* sequence.

[00149] 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. An isolated nucleic acid comprising a nucleotide sequence that encodes a mutant adeno-associated virus (AAV) capsid protein, wherein the mutant AAV capsid protein comprises at least one amino acid substitution relative to a wild-type AAV capsid protein.
2. The isolated nucleic acid of claim 1, wherein the encoded mutant AAV capsid protein exhibits increased heparan binding relative to a wild-type AAV capsid protein.
3. The isolated nucleic acid of claim 1, wherein the encoded mutant AAV capsid protein exhibits decreased heparan binding relative to a wild-type AAV capsid protein.
4. The isolated nucleic acid of claim 1, wherein the encoded mutant AAV capsid protein exhibits reduced binding to neutralizing antibody relative to a wild-type AAV capsid protein.
5. An isolated host cell comprising the nucleic acid of claim 1.
6. The isolated host cell of claim 5, wherein the host cell is stably transfected with the nucleic acid.
7. The isolated host cell of claim 5, further comprising a nucleic acid comprising a nucleotide sequence encoding an adeno-associated virus (AAV) rep protein.
8. The isolated host cell of claim 7, further comprising a recombinant AAV vector.
9. An adeno-associated virus (AAV) library, comprising a plurality of nucleic acids comprising a nucleotide sequence that encodes a mutant AAV capsid protein, wherein the mutant AAV capsid protein comprises at least one amino acid substitution relative to a wild-type AAV capsid protein.

10. A library of mutant adeno-associated virus (AAV) particles, comprising a plurality of AAV particles comprising an AAV capsid protein comprising at least one amino acid substitution relative to a wild-type AAV capsid protein.

11. A recombinant adeno-associated virus (rAAV) virion comprising an AAV capsid protein comprising at least one amino acid substitution relative to a wild-type AAV capsid protein; and a heterologous nucleic acid.

12. The rAAV virion of claim 11, wherein the rAAV virion exhibits reduced heparan binding relative to wild-type AAV.

13. The rAAV virion of claim 11, wherein the rAAV virion exhibits increased heparan binding relative to wild-type AAV.

14. The rAAV virion of claim 11, wherein the rAAV virion exhibits reduced inhibition of infection by neutralizing antibodies relative to the inhibition of infection by neutralizing antibodies of wild-type AAV virion.

15. The rAAV virion of claim 11, wherein the rAAV virion exhibits increased capacity to cross an endothelial cell layer relative to wild-type AAV virion.

16. The rAAV virion of claim 11, wherein the rAAV virion exhibits increased infectivity of a non-permissive cell relative to wild-type AAV virion.

17. A method of generating a mutant adeno-associated virus (AAV) particle comprising an AAV capsid protein comprising at least one amino acid substitution relative to a wild-type AAV capsid protein, wherein the mutant AAV particle exhibits an altered capsid property, the method comprising:

- a) generating a library of AAV mutants comprising a mutation in an AAV *cap* gene;
- b) packaging the AAV library into AAV particles, generating AAV library particles; and
- c) selecting the AAV library particles for an altered capsid property.

18. The method of claim 17, wherein generation of the library of AAV mutants comprises polymerase chain reaction mutagenesis.
19. The method of claim 18, where the mutagenesis comprises a staggered extension process.
20. The method of claim 17, wherein generation of the library of AAV mutants comprises oligonucleotide-directed mutagenesis.
21. The method of claim 17, wherein the selecting step comprises selecting for reduced binding to heparan.
22. The method of claim 17, wherein the selecting step comprises selecting for increased binding to heparan.
23. The method of claim 17, wherein the selecting step comprises selecting for reduced binding to neutralizing antibodies.
24. The method of claim 17, wherein the selecting step comprises selecting for increased infectivity of a non-permissive cell.
25. The method of claim 17, wherein the selecting step comprises selecting for increased ability to cross an endothelial cell layer.
26. A method of delivering a gene product to an individual in need thereof, the method comprising administering to the individual a recombinant adeno-associated virus (rAAV) virion according to claim 11.

MUTANT ADENO-ASSOCIATED VIRUS VIRIONS AND METHODS OF USE THEREOF

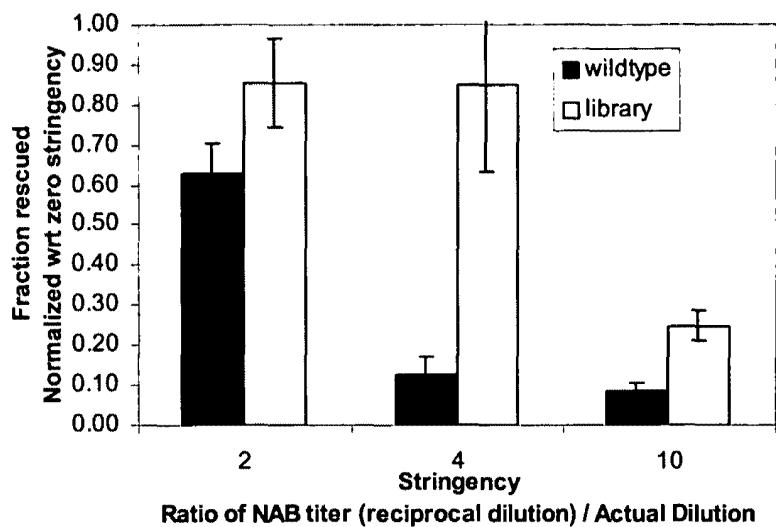
ABSTRACT OF THE DISCLOSURE

The present invention provides mutant adeno-associated virus (AAV) that exhibit altered capsid properties, e.g., reduced binding to neutralizing antibodies in serum and/or altered heparan binding and/or altered infectivity of particular cell types. The present invention further provides libraries of mutant AAV comprising one or more mutations in a capsid gene. The present invention further provides methods of generating the mutant AAV and mutant AAV libraries, and compositions comprising the mutant AAV. The present invention further provides recombinant AAV (rAAV) virions that comprise a mutant capsid protein. The present invention further provides nucleic acids comprising nucleotide sequences that encode mutant capsid proteins, and host cells comprising the nucleic acids. The present invention further provides methods of delivering a gene product to an individual, the methods generally involving administering an effective amount of a subject rAAV virion to an individual in need thereof.

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Figure 1

a)



b)

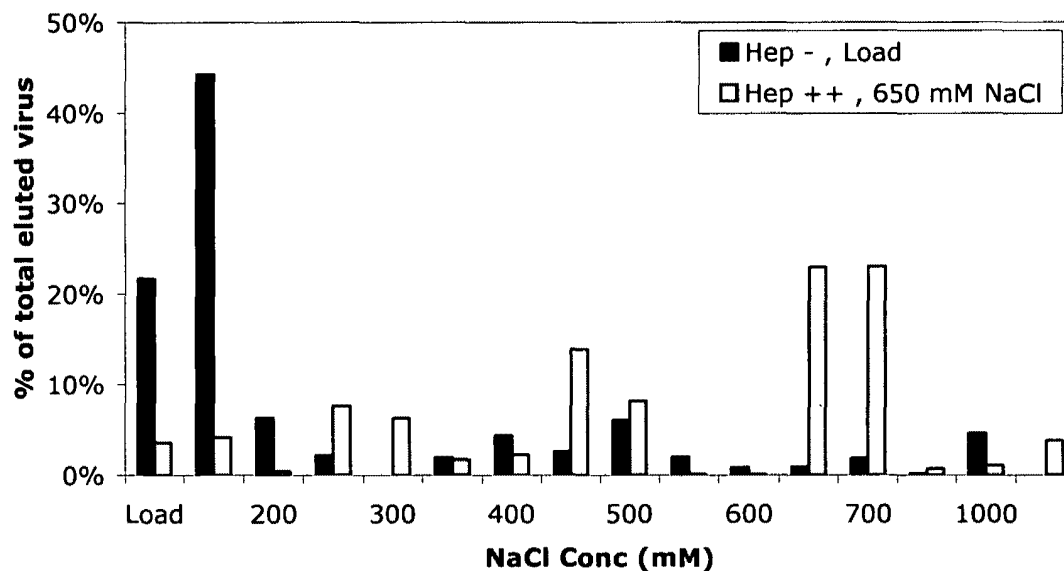
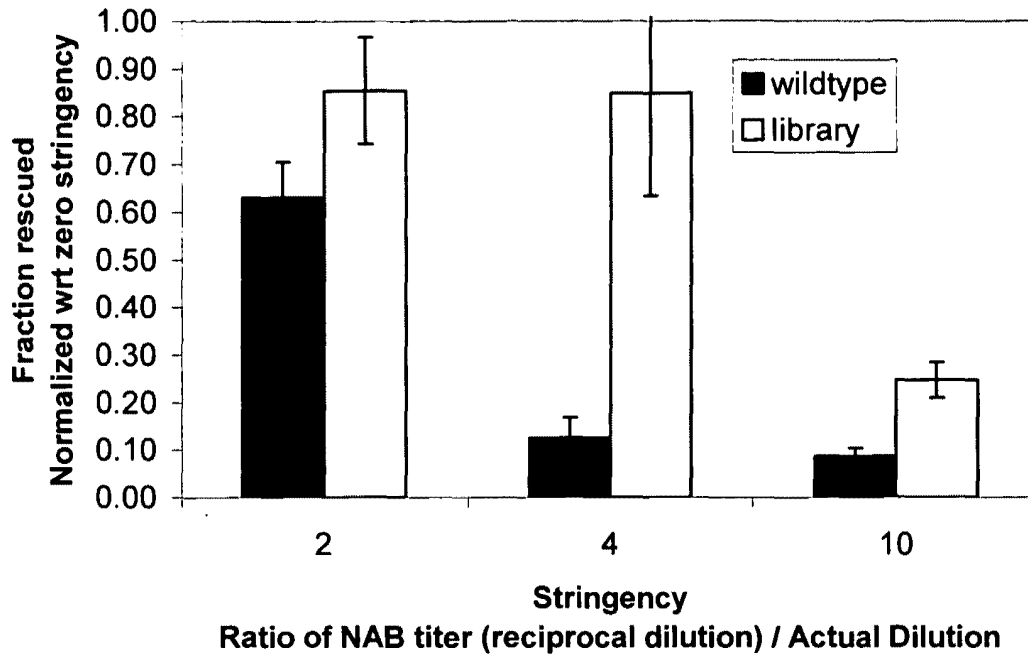


Figure 2
a)



b)

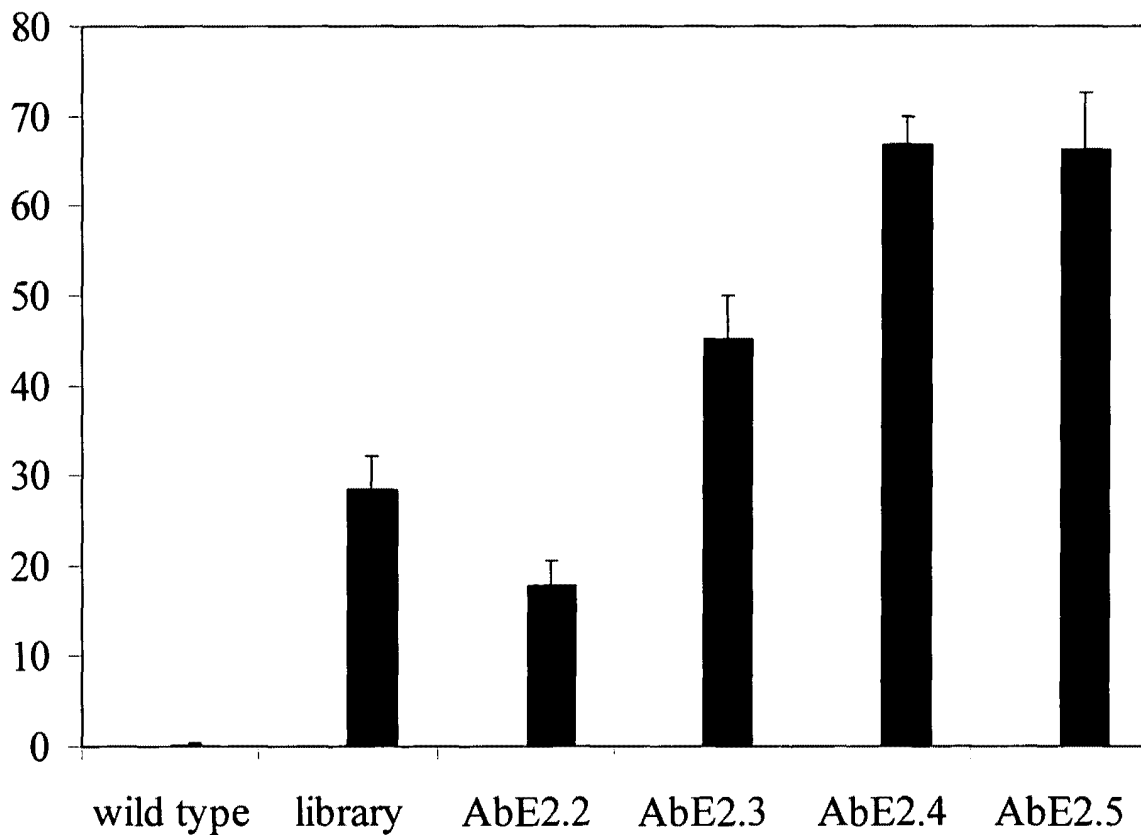


Figure 3i

wildtype CAP	1	atggctgccgatgggttatcttccagattggctcgaggacactctctctgaaggaataaga	60
AbE 2	1	ATGGCTGCCGATGGTTATCTTCCAGATTGGCTCGAGGACACTCTCTCTGAAGGAATAAGA	60
AbE L	1	ATGGCTGCCGATGGTTATCTTCCAGATTGGCTCGAGGACACTCTCTCTGAAGGAATAAGA	60
wildtype CAP	61	cagtgggtggaagctcaaacctggccccaccaccaccaaagcccgagagcggcataaggac	120
AbE 2	61	CAGTGGTGGAAGCTCAAACCTGGCCCCACCACCACCAAAGCCCGCAGAGCGGCATAAGGAC	120
AbE L	61	CAGTGGTGGAAGCTCAAACCTGGCCCCACCACCACCAAAGCCCGCAGAGCGGCATAAGGAC	120
wildtype CAP	121	gacagcaggggtcttgtgcttccctgggtacaagtacctcggacccttcaacggactcgac	180
AbE 2	121	GACAGCAGGGGTCTTGTGCTTCCCTGGGTACAAGTACCTCGGACCCTTCAACGGACTCGAC	180
AbE L	121	GACAGCAGGGGTCTTGTGCTTCCCTGGGTACAAGTACCTCGGACCCTTCAACGGACTCGAC	180
wildtype CAP	181	aaggagagccggtcaacgaggcagacgcgcggccctcgagcagcacaagcctacgac	240
AbE 2	181	AAGGGAGAGCCGGTCAACGAGGCAGACGCCGCGGCCCTCGAGCACGACAAAGCCTACGAC	240
AbE L	181	AAGGGAGAGCCGGTCAACGAGGCAGACGCCGCGGCCCTCGAGCACGACAAAGCCTACGAC	240
wildtype CAP	241	cggcagctcgacagcggagacaacccgtacctcaagtacaaccacgcgcgagcggagttt	300
AbE 2	241	CGGCAGCTCGACAGCGGAGACAACCCGTACCTCAAGTACAACCACGCCGACGCGGAGTTT	300
AbE L	241	CGGCAGCTCGACAGCGGAGACAACCCGTACCTCAAGTACAACCACGCCGACGCGGAGTTT	300
wildtype CAP	301	caggagcgccttaaagaagatacgtcttttgggggcaacctcggacgagcagtcctccag	360
AbE 2	301	CAGGAGCGCCTTAAAGAAGATACGTCTTTTGGGGGCAACCTCGGACGAGCAGTCTTCCAG	360
AbE L	301	CAGGAGCGCCTTAAAGAAGATACGTCTTTTGGGGGCAACCTCGGACGAGCAGTCTTCCAG	360
wildtype CAP	361	gcgaaaaagaggggttcttgaacctctgggcctgggttgaggaacctgttaagacggctccg	420
AbE 2	361	GCGAAAAAGAGGGTTCTTGAACCTCTGGGCCTGGTTGAGGAACCTGTTAAGACGGCTCCG	420
AbE L	361	GCGAAAAAGAGGGTTCTTGAACCTCTGGGCCTGGTTGAGGAACCTGTTAAGACGGCTCCG	420
wildtype CAP	421	ggaaaaaagagggccggtagagcactctcctgtggagccagactcctcctcgggaaccgga	480
AbE 2	421	GGAAAAAGAGGCCCGGTAGAGCACTCTCCTGTGGAGCCAGACTCCTCCTCGGGAACCGGA	480
AbE L	421	GGAAAAAGAGGCCCGGTAGAGCACTCTCCTGTGGAGCCAGACTCCTCCTCGGGAACCGGA	480
wildtype CAP	481	aaggcggggccagcagcctgcaagaaaaagattgaattttgggtcagactggagacgcagac	540
AbE 2	481	AAGGCGGGCCAGCAGCCTGCAAGAAAAAGATTGAATTTTGGTCAGACTGGAGACGCAGAC	540
AbE L	481	AAGGCGGGCCAGCAGCCTGCAAGAAAAAGATTGAATTTTGGTCAGACTGGAGACGCAGAC	540
wildtype CAP	541	tcagtacctgacccccagcctctcggacagccaccagcagccccctctgggtctgggaact	600
AbE 2	541	TCAGTACCTGACCCCCAGCCTCTCGGACAGCCACCAGCAGCCCCCTCTGGTCTGGGAACT	600
AbE L	541	TCAGTACCTGACCCCCAGCCTCTCGGACAGCCACCAGCAGCCCCCTCTGGTCTGGGAACT	600
wildtype CAP	601	aatacgatggctacaggcagtgggcgaccaatggcagacaataacgagggcgccgacgga	660
AbE 2	601	AATACGATGGCTACAGGCAGTGGCGCGCCAATGGCAGACAATAACGAGGGCGCCGACGGA	660
AbE L	601	AATACGATGGCTACAGGCAGTGGCGCGCCAATGGCAGACAATAACGAGGGCGCCGACGGA	660
wildtype CAP	661	gtgggtaattcctcgggaaattggcattgcgattccacatggatgggcgacagagtcac	720
AbE 2	661	GTGGGTAATTCTCTCGGGAAATTGGCATTGCGATTCCACATGGATGGGCGACAGAGTCATC	720
AbE L	661	GTGGGTAATTCTCTCGGGAAATTGGCATTGCGATTCCACATGGATGGGCGACAGAGTCATC	720
wildtype CAP	721	accaccagcaccggaacctggggcctgcccacctacaacaaccacctctacaacaaatt	780
AbE 2	721	ACCACCAGCACCCGAACCTGGGGCCTGCCCACCTACAACAACCACCTCTACAAACAAATT	780
AbE L	721	ACCACCAGCACCCGAACCTGGGGCCTGCCCACCTACAACAACCACCTCTACAAACAAATT	780
wildtype CAP	781	tccagccaatcaggagcctcgaacgacaatcactacttttggtacagcacccttggggg	840
AbE 2	781	TCCAGCCAATCAGGAGCCTCGAACGACAATCACTACTTTGGCTACAGCACCCCTTGGGGG	840
AbE L	781	TCCAGCCAATCAGGAGCCTCGAACGACAATCACTACTTTGGCTACAGCACCCCTTGGGGG	840
wildtype CAP	841	tattttgacttcaacagattccactgccacttttaccacgtgactggcaaagactcatc	900
AbE 2	841	TATTTTGACTTCAACAGATTCCACTGCCACTTTTACCACGTGACTGGCAAAGACTCATC	900
AbE L	841	TATTTTGACTTCAACAGATTCCACTGCCACTTTTACCACGTGACTGGCAAAGACTCATC	900

Figure 3ii

wildtype CAP	901	aacaacaactggggattccgacccaagagactcaacttcaagctctttaacattcaagtc	960
AbE 2	901	AACAACAAC TGGGGATTCCGACCCAAGAGACTCAACTTCAAGCTCTTTAATTC AAGTC	960
AbE L	901	AACAACAAC TGGGGATTCCGACCCAAGAGACTCAACTTCAAGCTCTTTAATTC AAGTC	960
wildtype CAP	961	aaagaggtcacgcagaatgacggtagcgacgacgattgccataaccttaccagcacgggt	1020
AbE 2	961	AAAGAGGT CACGCAGAATGACGGTAGC GACGACGATTGCCAATAACCTTACCAGCACGGTT	1020
AbE L	961	AAAGAGGT CACGCAGAATGACGGTAGC GACGACGATTGCCAATAACCTTACCAGCACGGTT	1020
wildtype CAP	1021	caggtgtttactgactcggagtagcagctcccgtacgtcctcgggtcggcgcatcaagga	1080
AbE 2	1021	CAGGTGTTTACTGACTCGGAGTACCAGCTCCCGTACGTCCTCGGCTCGGCGCATCAAGGA	1080
AbE L	1021	CAGGTGTTTACTGACTCGGAGTACCAGCTCCCGTACGTCCTCGGCTCGGCGCATCAAGGA	1080
wildtype CAP	1081	tgcctcccgcggttcccagcagacgtcttcatgggtgccacagtatggatacctcaccctg	1140
AbE 2	1081	TGCCTCCCGCGGTTCCCGAGCAGACGTCTTCATGGTGCCACAGTATGGATACCTCACCCTG	1140
AbE L	1081	TGCCTCCCGCGGTTCCCGAGCAGACGTCTTCATGGTGCCACAGTATGGATACCTCACCCTG	1140
wildtype CAP	1141	aacaacgggagtcaggcagtaggacgctcttcattttactgcctggagtagcttctcttct	1200
AbE 2	1141	AACAACGGGAGTCAGGCAGTAGGACGCTCTTCATTTTACTGCCTGGAGTACTTTCTCTTCT	1200
AbE L	1141	AACAACGGGAGTCAGGCAGTAGGACGCTCTTCATTTTACTGCCTGGAGTACTTTCTCTTCT	1200
wildtype CAP	1201	cagatgctgctgacggaacaaactttaccttcagctacacttttgaggacgttccctttc	1260
AbE 2	1201	CAGATGCTGCGTACCGGAAACAAC TTTACCTTCAGCTACACTTTTGAGGACCTTCCTTTTCT	1260
AbE L	1201	CAGATGCTGCGTACCGGAAACAAC TTTACCTTCAGCTACACTTTTGAGGACCTTCCTTTTCT	1260
wildtype CAP	1261	cacagcagctacgctcacagccagagtagtgacgctctcatgaatcctctcatcgaccag	1320
AbE 2	1261	CACAGCAGCTACGCTCACAGCCAGAGTCTGGACCGTCTCATGAATCCTCTCATCGACCAG	1320
AbE L	1261	CACAGCAGCTACGCTCACAGCCAGAGTCTGGACCGTCTCATGAATCCTCTCATCGACCAG	1320
wildtype CAP	1321	tacctgtattacttgagcagaacaaacactccaagtggaaaccaccacgcagtagcaggtt	1380
AbE 2	1321	TACCTGTATTACTTGAGCAGAACAACACTCCAAGTGGAACCACCACGCAGTCAAGGCTT	1380
AbE L	1321	TACCTGTATTACTTGAGCAGAACAACACTCCAAGTGGAACCACCACGCAGTCAAGGCTT	1380
wildtype CAP	1381	cagttttctcaggccggagcagtagcattcgggaccagtaggaactggcttctctgga	1440
AbE 2	1381	CAGTTTTCTCAGGCTGGAGCGAGTGACATTCGGGACCAGTCTAGGAAGTGGCTTCTCTGGA	1440
AbE L	1381	CAGTTTTCTCAGGCTGGAGCGAGTGACATTCGGGACCAGTCTAGGAAGTGGCTTCTCTGGA	1440
wildtype CAP	1441	ccctgttaccgccagcagcagtagtatcaaagacatctgctggataacaacaacagtagaatac	1500
AbE 2	1441	CCCTGTTACCGCCAGCAGAGAGTATCAAAGACATCTGCGGATAACAACAACAGTGAATAC	1500
AbE L	1441	CCCTGTTACCGCCAGCAGAGAGTATCAAAGACATCTGCGGATAACAACAACAGTGAATAC	1500
wildtype CAP	1501	tcgtggactggagctaccaagtagcacctcaatggcagagactctctggtagaatccgggc	1560
AbE 2	1501	TCGTGGACTGGAGCTACCAAGTACCACCTCAATGGCAGAGACTCTCTGGTGAATCCGGGC	1560
AbE L	1501	TCGTGGACTGGAGCTACCAAGTACCACCTCAATGGCAGAGACTCTCTGGTGAATCCGGGC	1560
wildtype CAP	1561	ccggccatggcaagccacaaggacgatgaagaaaagttttttctcagagcgggggttctc	1620
AbE 2	1561	CCGGCCATGGCAAGCCACAAGGACGATGAAGAAAAGTTTTTCTCAGAGCGGGGTCTCTC	1620
AbE L	1561	CCGGCCATGGCAAGCCACAAGGACGATGAAGAAAAGTTTTTCTCAGAGCGGGGTCTCTC	1620
wildtype CAP	1621	atctttgggaagcaaggctcagagaaaacaaatgtggacattgaaaaggtagatgattaca	1680
AbE 2	1621	ATCTTTGGGAAGCAAGGCTCAGAGAAAACAAATGTGGACATTGAAAAGGTCTGATTACA	1680
AbE L	1621	ATCTTTGGGAAGCAAGGCTCAGAGAAAACAAATGTGGACATTGAAAAGGTCTGATTACA	1680
wildtype CAP	1681	gacgaagaggaaatcaggacaaccaatcccgtggctacggagcagtaggttctgtatct	1740
AbE 2	1681	GACGAAGAGGAAATCAGGACAACCAATCCCGTGGCTACGGAGCAGTATGGTTCTGTATCT	1740
AbE L	1681	GACGAAGAGGAAATCAGGACAACCAATCCCGTGGCTACGGAGCAGTATGGTTCTGTATCT	1740
wildtype CAP	1741	accaacctccagagaggcaacagacaagcagtagcagtagtcaacacacaaggcggt	1800
AbE 2	1741	ACCAACCTCCAGAGAGGCAACAGACAAGCAGCTACCGCAGATGTCAACACACAAGGCGTT	1800
AbE L	1741	ACCAACCTCCAGAGAGGCAACAGACAAGCAGCTACCGCAGATGTCAACACACAAGGCGTT	1800

Figure 3iii

wildtype CAP	1801	cttccaggcatggtctggcaggacagagatgtgtaccttcaggggcccacatctgggcaaag	1860
AbE 2	1801	CTTCCAGGCATGGTCTGGCAGGACAGAGATGTGTACCTTCAGGGGCCCACATCTGGGCAAAG	1860
AbE L	1801	CTTCCAGGCATGGTCTGGCAGGACAGAGATGTGTACCTTCAGGGGCCCACATCTGGGCAAAG	1860
wildtype CAP	1861	attccacacacggacggacattttcaccctctccccctcatgggtggattcggacttaaa	1920
AbE 2	1861	ATTCCACACACGGACGGACATTTTCACCCCTCTCCCCTCATGGGTGGATTTCGGACTTAAA	1920
AbE L	1861	ATTCCACACACGGACGGACATTTTCACCCCTCTCCCCTCATGGGTGGATTTCGGACTTAAA	1920
wildtype CAP	1921	caccctcctccacagattctcatcaagaacaccccggtacctgcgaatccttcgaccacc	1980
AbE 2	1921	CACCCTCCTCCACAGATTCTCATCAAGAACACCCCGGTACCTGCGAATCCTTCGACCACC	1980
AbE L	1921	CACCCTCCTCCACAGATTCTCATCAAGAACACCCCGGTACCTGCGAATCCTTCGACCACC	1980
wildtype CAP	1981	ttcagtgcggaaggtttgcttccttcacacagtagtccacgggacaggtcagcgtg	2040
AbE 2	1981	TTCAGTGCGGCAAAGTTTGCTTCCTTCATCACACAGTACTCCACGGGACAGGTTCAGCGTG	2040
AbE L	1981	TTCAGTGCGGCAAAGTTTGCTTCCTTCATCACACAGTACTCCACGGGACAGGTTCAGCGTG	2040
wildtype CAP	2041	gagatcgagtgaggagctgcagaaggaaaacagcaaacgctggaatcccgaaattcagtag	2100
AbE 2	2041	GAGATCGAGTGGGAGCTGCAGAAGGAAAACAGCAAACGCTGGAATCCCGAAATTCAGTAC	2100
AbE L	2041	GAGATCGAGTGGGAGCTGCAGAAGGAAAACAGCAAACGCTGGAATCCCGAAATTCAGTAC	2100
wildtype CAP	2101	acttccaactacaacaagtctgttaatgtggactttactgtggacactaatggcgtgtat	2160
AbE 2	2101	ACTTCCAACACTACAACAAGTCTGTTAATGTGGACTTTAGGATGGACGCTAATGGCGTGTAT	2160
AbE L	2101	ACTTCCAACACTACAACAAGTCTGTTAATGTGGACTTTACNGTGGACGCTAATGGCGTGTAT	2160
wildtype CAP	2161	tcagagcctcgccccattggcaccagatacctgactcgtaattctgtaattgcttgcggcc	2220
AbE 2	2161	TCAGAGCCNNNCCCCATTGGCACCAGT-----	2187
AbE L	2161	TCAGAGCCNNGCCCCATTGGCACCAGT-----	2187
wildtype CAP	2221	gcccc	2225
AbE 2	2187	-----	2187
AbE L	2187	-----	2187

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CONTINUITY INFORMATION

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Filing Date::

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Filing Date::

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which is a::
>>> Application Four::
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