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(72) Feltaláló(k): Arbetman, Alejandra Elena, San Francisco, CA 94122 (US) Colosi, Peter, Alameda, CA 94501 (US) Lochrie, Michael A., Hayward, CA 94541 (US) Surosky, Richard T., Oakland, CA 94618 (US)	(74) Képviselő: Danubia Szabadalmi és Jogi Iroda Kft., Budapest

(54) **Csökkentett immunreaktivitású AAV virionok és alkalmazásai**

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(54) **AAV virions with decreased immunoreactivity and uses therefor**

AAV-Virionen mit verminderter Immunreaktivität und Anwendungen dafür

Virions AAV présentant une immunoreactivité réduite et leurs utilisations

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(73) Proprietor: **Genzyme Corporation**

Cambridge, Massachusetts 02142 (US)

(72) Inventors:

- **Arbetman, Alejandra Elena**
San Francisco, CA 94122 (US)
- **Colosi, Peter**
Alameda, CA 94501 (US)
- **Lochrie, Michael A.**
Hayward, CA 94541 (US)
- **Suroskey, Richard T.**
Oakland, CA 94618 (US)

(74) Representative: **Adams, Harvey Vaughan John**

Mathys & Squire LLP
The Shard
32 London Bridge Street
London SE1 9SG (GB)

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Description**TECHNICAL FIELD**

5 **[0001]** The present disclosure relates generally to compositions and methods for delivering recombinant adeno-associated virus (rAAV) virions to cells. In particular, the present disclosure pertains to rAAV virions with decreased immunoreactivity (mutant rAAV virions) and methods of making and using the same.

BACKGROUND

10 **[0002]** Scientists are continually discovering genes that are associated with human diseases such as diabetes, hemophilia, and cancer. Research efforts have also uncovered genes, such as erythropoietin (which increases red blood cell production), that are not associated with genetic disorders but instead code for proteins that can be used to treat numerous diseases. Despite significant progress in the effort to identify and isolate genes, however, a major obstacle
15 facing the biopharmaceutical industry is how to safely and persistently deliver therapeutically effective quantities of gene products to patients.

20 **[0003]** Generally, the protein products of these genes are synthesized in cultured bacteria, yeast, insect, mammalian, or other cells and delivered to patients by direct injection. Injection of recombinant proteins has been successful but suffers from several drawbacks. For example, patients often require weekly, and sometimes daily, injections in order to maintain the necessary levels of the protein in the bloodstream. Even then, the concentration of protein is not maintained at physiological levels—the level of the protein is usually abnormally high immediately following the injection, and far below optimal levels prior to the injection. Additionally, injected delivery of recombinant protein often cannot deliver the protein to the target cells, tissues, or organs in the body. And, if the protein successfully reaches its target, it may be diluted to a non-therapeutic level. Furthermore, the method is inconvenient and often restricts the patient's lifestyle.

25 **[0004]** These shortcomings have fueled the desire to develop gene therapy methods for delivering sustained levels of specific proteins into patients. These methods are designed to allow clinicians to introduce deoxyribonucleic acid (DNA) coding for a nucleic acid, such as a therapeutic gene, directly into a patient (*in vivo* gene therapy) or into cells isolated from a patient or a donor (*ex vivo* gene therapy). The introduced nucleic acid then directs the patient's own cells or grafted cells to produce the desired protein product. Gene delivery, therefore, obviates the need for frequent injections.
30 Gene therapy may also allow clinicians to select specific organs or cellular targets (e.g., muscle, blood cells, brain cells, etc.) for therapy.

35 **[0005]** DNA may be introduced into a patient's cells in several ways. There are transfection methods, including chemical methods such as calcium phosphate precipitation and liposome-mediated transfection, and physical methods such as electroporation. In general, transfection methods are not suitable for *in vivo* gene delivery. There are also methods that use recombinant viruses. Current viral-mediated gene delivery vectors include those based on retrovirus, adenovirus, herpes virus, pox virus, and adeno-associated virus (AAV). Like the retroviruses, and unlike adenovirus, AAV has the ability to integrate its genome into a host cell chromosome.

Adeno-Associated Virus-Mediated Gene Therapy

40 **[0006]** AAV is a parvovirus belonging to the genus Dependovirus, and has several attractive features not found in other viruses. For example, AAV can infect a wide range of host cells, including non-dividing cells. AAV can also infect cells from different species. Importantly, AAV has not been associated with any human or animal disease, and does not appear to alter the physiological properties of the host cell upon integration. Furthermore, AAV is stable at a wide range
45 of physical and chemical conditions, which lends itself to production, storage, and transportation requirements.

50 **[0007]** The AAV genome, a linear, single-stranded DNA molecule containing approximately 4700 nucleotides (the AAV-2 genome consists of 4681 nucleotides), generally comprises an internal non-repeating segment flanked on each end by inverted terminal repeats (ITRs). The ITRs are approximately 145 nucleotides in length (AAV-1 has ITRs of 143 nucleotides) and have multiple functions, including serving as origins of replication, and as packaging signals for the viral genome.

55 **[0008]** The internal non-repeated portion of the genome includes two large open reading frames (ORFs), known as the AAV replication (*rep*) and capsid (*cap*) regions. These ORFs encode replication and capsid gene products, respectively: replication and capsid gene products (i.e., proteins) allow for the replication, assembly, and packaging of a complete AAV virion. More specifically, a family of at least four viral proteins are expressed from the AAV *rep* region: Rep 78, Rep 68, Rep 52, and Rep 40, all of which are named for their apparent molecular weights. The AAV *cap* region encodes at least three proteins: VP1, VP2, and VP3.

60 **[0009]** In nature, AAV is a helper virus-dependent virus, i.e., it requires co-infection with a helper virus (e.g., adenovirus, herpesvirus, or vaccinia virus) in order to form functionally complete AAV virions. In the absence of co-infection with a

helper virus, AAV establishes a latent state in which the viral genome inserts into a host cell chromosome or exists in an episomal form, but infectious virions are not produced. Subsequent infection by a helper virus "rescues" the integrated genome, allowing it to be replicated and packaged into viral capsids, thereby reconstituting the infectious virion. While AAV can infect cells from different species, the helper virus must be of the same species as the host cell. Thus, for example, human AAV will replicate in canine cells that have been co-infected with a canine adenovirus.

[0010] To construct infectious recombinant AAV (AAV) containing a nucleic acid, a suitable host cell line is transfected with an AAV vector containing a nucleic acid. AAV helper functions and accessory functions are then expressed in the host cell. Once these factors come together, the HNA is replicated and packaged as though it were a wild-type (wt) AAV genome, forming a recombinant virion. When a patient's cells are infected with the resulting rAAV, the HNA enters and is expressed in the patient's cells. Because the patient's cells lack the *rep* and *cap* genes, as well as the adenovirus accessory function genes, the rAAV are replication defective; that is, they cannot further replicate and package their genomes. Similarly, without a source of *rep* and *cap* genes, wtAAV cannot be formed in the patient's cells.

[0011] There are several AAV serotypes that infect humans as well as other primates and mammals. Eight major serotypes have been identified, AAV-1 through AAV-8, including two serotypes recently isolated from rhesus monkeys. Gao et al. (2002) Proc. Natl. Acad. Sci. USA 99:11854-11859. Of those serotypes, AAV-2 is the best characterized, having been used to successfully deliver transgenes to several cell lines, tissue types, and organs in a variety of *in vitro* and *in vivo* assays. The various serotypes of AAV can be distinguished from one another using monoclonal antibodies or by employing nucleotide sequence analysis; e.g., AAV-1, AAV-2, AAV-3, and AAV-6 are 82% identical at the nucleotide level, while AAV-4 is 75 to 78% identical to the other serotypes (Russell et al. (1998) J. Virol. 72:309-319). Significant nucleotide sequence variation is noted for regions of the AAV genome that code for capsid proteins. Such variable regions may be responsible for differences in serological reactivity to the capsid proteins of the various AAV serotypes.

[0012] After an initial treatment with a given AAV serotype, anti-AAV capsid neutralizing antibodies are often made which prevent subsequent treatments by the same serotype. For example, Moskalenko et al. J. Virol. (2000) 74:1761-1766 showed that mice with pre-existing anti-AAV-2 antibodies, when administered Factor IX in a recombinant AAV-2 virion, failed to express the Factor IX transgene, suggesting that the anti-AAV-2 antibodies blocked transduction of the rAAV-2 virion. Halbert et al. J. Virol. (1998) 72:9795-9805 reported similar results. Others have demonstrated successful readministration of rAAV-2 virions into experimental animals, but only after immune suppression is achieved (see, e.g., Halbert et al., *supra*).

[0013] Thus, using rAAV for human gene therapy is potentially problematic because anti-AAV antibodies are prevalent in human populations. Infection of humans by a variety of AAV serotypes occurs in childhood, and possibly even *in utero*. In fact, one study estimated that at least 80% of the general population has been infected with AAV-2 (Berns and Linden (1995) Bioessays 17:237-245). Neutralizing anti-AAV-2 antibodies have been found in at least 20-40 % of humans. Our studies have shown that out of a group of 50 hemophiliacs, approximately 40% had AAV-2 neutralizing capacities exceeding 1e13 viral particles/ml, or about 6e16 viral particles/total blood volume. Furthermore, the majority of the group with high anti-AAV-2 titers also had significant titers against other AAV serotypes, such as AAV-1, AAV-3, AAV-4, AAV-5 and AAV-6. Therefore, identification of AAV mutants with reduced immunoreactivity, such as mutants that are not neutralized by pre-existing anti-AAV, antibodies, would be a significant advancement in the art. Such AAV mutants are described herein.

[0014] Wu et al. (2000) J. Virology, Vol. 74, No. 18, 8635-8647 states that the authors have constructed 93 mutants at 59 different positions in the AAV capsid gene by site directed mutagenesis in order to obtain a comprehensive genetic map of the AAV gene.

SUMMARY OF THE INVENTION

[0015] The present disclosure is based on the discovery of novel AAV sequences, such as mutated AAV sequences, that provide for recombinant AAV virions with decreased immunoreactivity as compared with the corresponding native serotype but which retain the ability to efficiently transduce cells and tissues. The rAAV virions with decreased immunoreactivity are especially useful for delivering heterologous nucleic acid molecules (HNAs) to subjects that have been previously exposed to AAV, either by natural infection or due to previous gene therapy or nucleic acid Immunization treatments, and have therefore developed anti-AAV antibodies. The rAAV virions described herein are therefore useful for treating or preventing a wide variety of disorders, as described further below, in vertebrate subjects that have been previously exposed to any of the various AAV serotypes. In accordance with the present disclosure, then, methods and AAV vectors for use therein are provided for the efficient delivery of HNAs to the cells or tissue of a vertebrate subject, such as a mammal, using recombinant AAV virions.

[0016] In certain instances, the present disclosure provides for the use of AAV virions containing altered capsid proteins to deliver an HNA encoding antisense RNA, ribozymes, or one or more genes that express proteins, wherein expression of Said antisense RNA, ribozymes, or one or more genes provides for a biological effect in a mammalian subject. In one instance, the rAAV virions containing an HNA are injected directly into a muscle (e.g., cardiac, smooth and/or skeletal

muscle). In another embodiment, the rAAV virions containing an HNA are administered into the vasculature via injection into veins, arteries, or other vascular conduits, or by using techniques such as isolated limb perfusion.

[0017] In additional instances, the virions contain a gene encoding a blood coagulation protein which, when expressed at a sufficient concentration, provides for a therapeutic effect, such as improved blood-clotting efficiency of a mammal buffering from a blood-clotting disorder. The blood-clotting disorder can be any disorder adversely affecting the organism's ability to coagulate the blood. Preferably, the blood clotting disorder is hemophilia. In one instance, then, the gene encoding a blood coagulation protein is a Factor VIII gene, such as the human Factor VIII gene or a derivation thereof. In another instance, the gene encoding a blood coagulation protein is a Factor IX gene, such as the human Factor IX (hF.IX) gene.

[0018] Accordingly, in one instance, the present disclosure is directed to a mutated AAV capsid protein that when present in an AAV virion imparts decreased immunoreactivity to the virion as compared to the corresponding wild-type virion. The mutation may comprise at least one amino acid substitution, deletion or insertion to the native protein, such as a substitution is in the spike or plateau region of the AAV virion surface.

[0019] In certain instances, the amino acid substitution comprises a substitution of one or more of the amino acids occurring at a position corresponding to a position of the AAV-2 VP2 capsid selected from the group consisting of amino acid 126, 127, 128, 130, 132, 134, 247, 248, 315, 334, 354, 357, 360, 361, 365, 372, 375, 377, 390, 393, 394, 395, 396, 407, 411, 413, 418, 437, 449, 450, 568, 569, and 571. In additional instances, the naturally occurring amino acid at one or more of these positions is substituted with an alanine. In further instances, the protein further comprises a substitution of histidine for the amino acid occurring at the position corresponding to the amino acid found at position 360 of AAV-2 VP2 and/or a substitution of lysine for the amino acid occurring at the position corresponding to the amino acid found at position 571 of AAV-2 VP2.

[0020] In additional instances, the disclosure is directed to a polynucleotide encoding any of the mutated proteins described above.

[0021] In further instances, the disclosure is directed to a recombinant AAV virion comprising any of the mutated proteins described above. The recombinant AAV virion can comprise a heterologous nucleic acid molecule encoding an antisense RNA or a ribozymes, or a heterologous nucleic acid molecule encoding a therapeutic protein operably linked to control elements capable of directing the *in vivo* transcription and translation of said protein.

[0022] In yet further instances, the disclosure is directed to a method of delivering a recombinant AAV virion to a cell or tissue of a vertebrate subject. The method comprises:

- (a) providing a recombinant AAV virion as above;
- (b) delivering the recombinant AAV virion to the cell or tissue, whereby the protein is expressed at a level that provides a therapeutic effect.

[0023] In certain instances, the cell or tissue is a muscle cell or tissue. The muscle cell or tissue can be derived from skeletal muscle.

[0024] In further instances, the recombinant AAV virion is delivered into the cell or tissue *in vivo*.

[0025] In certain instances, the recombinant AAV virion is delivered by intramuscular injection, or into the bloodstream, such as intravenously or intraarterially. In additional embodiments, the recombinant AAV virion is delivered to the liver or to the brain.

[0026] In further instances, the recombinant AAV virion is delivered into said cell or tissue *in vitro*.

[0027] In yet additional instances, the disclosure is directed to a method of delivering a recombinant AAV virion to a cell or tissue of a vertebrate subject. The method comprises:

- (a) providing a recombinant AAV virion, wherein the AAV virion comprises
 - (i) a non-primate, mammalian adeno-associated virus (AAV) capsid protein that when present in an AAV virion imparts decreased immunoreactivity to the virion as compared to immunoreactivity of primate AAV-2; and
 - (ii) a heterologous nucleic acid molecule encoding a therapeutic protein operably linked to control elements capable of directing the *in vivo* transcription and translation of the protein;
- (b) delivering the recombinant AAV virion to the cell or tissue, whereby the protein is expressed at a level that provides a therapeutic effect.

[0028] In certain instances, the cell or tissue is a muscle cell or tissue, such as a muscle cell or tissue is derived from skeletal muscle.

[0029] The recombinant AAV virion is delivered into said cell or tissue *in vivo* or *in vitro* and can be delivered to the subject by intramuscular injection, or into the bloodstream, such as intravenously or intraarterially. In additional instances,

the recombinant AAV virion is delivered to the liver or to the brain.

[0030] Based on the present disclosure, a first aspect of the present invention provides for the use of a mutated adeno-associated virus (AAV) capsid protein, within a recombinant AAV virion, for decreasing the immunoreactivity of the virion as compared to the corresponding wild-type virion, wherein said mutated AAV capsid protein comprises a substitution of the amino acid which occurs at the position which corresponds to position 334 of the AAV VP2 capsid protein.

[0031] Further aspects and embodiments of the invention are set forth in the appended claims.

BRIEF DESCRIPTION OF THE FIGURES

[0032]

Figure 1 illustrates the location of an asymmetrical structural unit (white triangle) of AAV-2 on the surface of the entire virus (taken from Fig 3a of Xie et al. Proc. Natl. Acad. Sci. USA (2002) 99:10405-10410). There are 60 identical asymmetric structural units per AAV virion. At least 145 amino acids out of a total of 735 in each AAV-2 capsomere are exposed, to varying degrees, on the surface.

Figure 2 illustrates the location of some of the amino acids that were mutated as described in the examples within an asymmetric unit (black triangle) of the AAV-2 structure. The amino acids that were mutated are shown as black space-filling models, while those that were not mutated are shown as white stick models. The location of major surface features (spike, cylinder, plateau, canyon) is indicated and the approximate boundaries of these features are shown by thin circular black lines. The "canyon" regions, predicted to be relatively inaccessible to antibody binding, are located in the areas between the spike, cylinder, and plateau. The numbers 2, 3 and 5 represent the 2-, 3-, and 5-fold axes of symmetry, respectively.

Figure 3 indicates the location of mutations that have <10-fold effect on *in vitro* transduction. Mutations located at black space-filling amino acids, < 10% wild type transduction. The numbers 2, 3 and 5 represent 2-, 3- and 5-fold axes of symmetry, respectively.

Figure 4 indicates the location of mutations that have >10-fold effect on *in vitro* transduction. Mutations located at black space-filling amino acids, < 10% wild type transduction. The numbers 2, 3 and 5 represent 2-, 3- and 5-fold axes of symmetry, respectively. The approximate boundaries of two dead zones spanning the 2-fold axis of symmetry is indicated.

Figure 5 illustrates the location of some of the AAV-2 capsid mutants defective in heparin binding. Black amino acids designate heparin-defective mutants identified herein. Black amino acids illustrated as space-filling models (347, 350, 356, 375, 395, 448, 451) are on the surface. Grey amino acids illustrated as space-filling models (495, 592) are just under the surface. The numbers 2, 3 and 5 represent the 2-, 3- and 5-fold axes of symmetry, respectively. Mutants that have more than a 100-fold effect on heparin binding are enclosed in circles.

Figure 6 illustrates the location of some of the amino acids (black space-filling model) on the surface of the AAV-2 capsid that confer resistance to neutralization by a mouse monoclonal antibody when they are individually mutated. The rectangular box represents the approximate size of an antibody binding site (25 Å x 35 Å). The numbers 2, 3, and 5 represent the 2-, 3- and 5-fold axes of symmetry, respectively.

Figure 7 illustrates the location of some of the amino acids (black space-filling model) on the surface of the AAV-2 capsid that confer resistance to neutralization by multiple human antisera. The rectangular box represents the approximate size of an antibody binding site (25 Å x 35 Å). The numbers 2, 3, and 5 represent the 2-, 3- and 5-fold axes of symmetry, respectively.

Figure 8 shows mouse monoclonal antibody titration properties of four AAV-2 capsid mutants compared to AAV-2 with a wild-type capsid.

Figure 9 shows the amino acid sequence of an AAV-2 VP2 (SEQ ID NO:12).

Figure 10 shows the amino acid sequence of an AAV-2 VP1 (SEQ ID NO:13).

Figure 11 shows the relative positions of AAV-2 capsid proteins VP1, VP2 and VP3. As shown in the figure, VP1, VP2 and VP3 share the same 533 C-terminal amino acids which make up VP3. As shown in the Figure, all capsid mutants described herein fall within the shared area.

Figures 12A-12B show a comparison of the nucleotide sequence encoding the AAV VP1 protein from a primate AAV-5 (SEQ ID NO:14) and a caprine AAV (SEQ ID NO:15). Numbering is relative to the AAV-5 full-length sequence. Figure 13 shows a comparison of the amino acid sequence of VP1 from a primate AAV-5 (SEQ ID NO:16) and a caprine AAV (SEQ ID NO:17). Amino acid differences are shaded. Conservative changes are shown in light grey; non-conservative changes are shown in dark grey.

Figures 14A-14H show a comparison of the amino acid sequence of VP1s from AAVs that are sensitive or resistant to antibody neutralization as follows: primate AAV-2 (SEQ ID NO:13), primate AAV-3B (SEQ ID NO:18), primate AAV-6 (SEQ ID NO:19), primate AAV-1 (SEQ ID NO:20), primate AAV-8 (SEQ ID NO:21), primate AAV-4 (SEQ ID NO:22), primate AAV-5 (SEQ ID NO:16) and caprine (goat) AAV (SEQ ID NO:17). Parvovirus line: *, conserved in

almost all parvoviruses. Neutralization line: #, location of single mutations in AAV-2 capsid identified as resistant to neutralization by human sera. Accessibility line: B, amino acid is buried between the inside and outside surface; I, amino acid is found on the inside surface; O, amino acid is found on the outside surface. Surface feature line: C, cylinder; P, plateau; S, spike; Y, canyon. DNA line: B, possible base contact; D, likely required for DNA binding but may not directly contact DNA; P, possible phosphate contact; R, possible ribose contact. Other line: A, location of single mutations that decrease binding and neutralization by mouse monoclonal antibody A20; H, heparin contact in AAV-2; M, possible Mg²⁺ contact; P, phospholipase A2 domain.

Figure 15 (SEQ ID NOS: 16 and 17) shows the positions of the amino acid differences between AAV-5 and caprine AAV, relative to the surface of the AAV capsid.

Figure 16 shows the predicted location of the surface amino acids that differ between AAV-5 and caprine AAV, based on the surface structure of the AAV-2 capsid. The 3 filled triangles represent insertions in caprine AAV, relative to AAV-2, that are likely to be located on the surface.

Figure 17 shows transduction of muscle in IVIG-treated SCID mice following intramuscular administration of various rAAV hFIX virions.

Figure 18 shows transduction of liver in IVIG-treated SCID mice following tail vein administration of various rAAV hFIX virions.

Figure 19 shows the biodistribution of human factor IX (hFIX) follow intravenous administration of a recombinant caprine AAV vector encoding the same.

Figures 20A (SEQ ID NO:25) and 20B (SEQ ID NO:26) show the nucleotide sequence and amino acid sequence respectively, of a bovine AAV VP1, from AAV-C1.

Figures 21A-21H show a comparison of the amino acid sequence of VP1s from AAVs that are sensitive or resistant to antibody neutralization as follows: primate AAV-2 (SEQ ID NO:13), primate AAV-3B (SEQ ID NO:18), primate AAV-6 (SEQ ID NO:19), primate AAV-1 (SEQ ID NO:20), primate AAV-8 (SEQ ID NO:21), primate AAV-4 (SEQ ID NO:22), bovine (cow) AAV ("AAV-C1" (SEQ ID NO:26), primate AAV-5 (SEQ ID NO:16) and caprine (goat) AAV ("AAV-C1" SEQ ID NO:17). Parvovirus line: *, conserved in almost all parvoviruses. Neutralization line: #, location of single mutations in AAV-2 capsid identified as resistant to neutralization by human sera. Accessibility line: B, amino acid is buried between the inside and outside surface; I, amino acid is found on the inside surface; O, amino acid is found on the outside surface. Surface feature line: C, cylinder; P, plateau; S, spike; Y, canyon. DNA line: B, possible base contact; D, likely required for DNA binding but may not directly contact DNA; P, possible phosphate contact; R, possible ribose contact. Other line: A, location of single mutations that decrease binding and neutralization by mouse monoclonal antibody A20; H, heparin contact in AAV-2; M, possible Mg²⁺ contact; P, phospholipase A2 domain.

DETAILED DESCRIPTION OF THE INVENTION

[0033] The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, recombinant DNA techniques and immunology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Fundamental Virology, 2nd Edition, vol. I & II (B.N. Fields and D.M. Knipe, eds.); Handbook of Experimental Immunology, Vols.I-IV (D.M. Weir and C.C. Blackwell eds., Blackwell Scientific Publications); T.E. Creighton, Proteins: Structures and Molecular Properties (W.H. Freeman and Company, 1993); A.L. Lehninger, Biochemistry (Worth Publishers, Inc., current addition); Sambrook, et al., Molecular Cloning: A Laboratory Manual (2nd Edition, 1989); Methods In Enzymology (S. Colowick and N. Kaplan eds., Academic Press, Inc.).

1. DEFINITIONS

[0034] In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

[0035] It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "a polypeptide" includes a mixture of two or more polypeptides, and the like.

[0036] The following amino acid abbreviations are used throughout the text:

Alanine: Ala (A)	Arginine: Arg (R)
Asparagine: Asn (N)	Aspartic acid: Asp (D)
Cysteine: Cys (C)	Glutamine: Gln (Q)
Glutamic acid: Glu (E)	Glycine: Gly (G)
Histidine: His (H)	Isoleucine: Ile (I)

(continued)

Leucine: Leu (L)	Lysine: Lys (K)
Methionine: Met (M)	Phenylalanine: Phe (F)
Proline: Pro (P)	Serine: Ser (S)
Threonine: Thr (T)	Tryptophan: Trp (W)
Tyrosine: Tyr (Y)	Valine: Val (V)

[0037] By "vector" is meant any genetic element, such as a plasmid, phage, transposon, cosmid, chromosome, virus, virion, etc., which is capable of replication when associated with the proper control elements and which can transfer gene sequences between cells. Thus, the term includes cloning and expression vehicles, as well as viral vectors.

[0038] By an "AAV vector" is meant a vector derived from any adeno-associated virus serotype isolated from any animal species, including without limitation, AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAV-8, AAV-G1 and AAV-C1. AAV vectors can have one or more of the AAV wild-type genes deleted in whole or part, preferably the *rep* and/or *cap* genes, but retain functional flanking ITR sequences. Functional ITR sequences are necessary for the rescue, replication and packaging of the AAV virion. Thus, an AAV vector is defined herein to include at least those sequences required in *cis* for replication and packaging (e.g., functional ITRs) of the virus. The ITRs need not be the wild-type nucleotide sequences, and may be altered, e.g., by the insertion, deletion or substitution of nucleotides, so long as the sequences provide for functional rescue, replication and packaging.

[0039] "AAV helper functions" refer to AAV-derived coding sequences which can be expressed to provide AAV gene products that, in turn, function in *trans* for productive AAV replication. Thus, AAV helper functions include both of the major AAV open reading frames (ORFs), *rep* and *cap*. The Rep expression products have been shown to possess many functions, including, among others: 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 Cap expression products supply necessary packaging functions. AAV helper functions are used herein to complement AAV functions in *trans* that are missing from AAV vectors.

[0040] The term "AAV helper construct" refers generally to a nucleic acid molecule that includes nucleotide sequences providing AAV functions deleted from an AAV vector which is to be used to produce a transducing vector for delivery of a nucleotide sequence of interest. AAV helper constructs are commonly used to provide transient expression of AAV *rep* and/or *cap* genes to complement missing AAV functions that are necessary for lytic AAV replication; however, helper constructs lack AAV ITRs and can neither replicate nor package themselves. AAV helper constructs can be in the form of a plasmid, phage, transposon, cosmid, virus, or virion. A number of AAV helper constructs and vectors that encode Rep and/or Cap expression products have been described. See, e.g., U.S. Patent Nos. 6,001,650, 5,139,941 and 6,376,237; Samulski et al. (1989) J. Virol. 63:3822-3828; and McCarty et al. (1991) J. Virol. 65:2936-2945.

[0041] The term "accessory functions" refers to non-AAV derived viral and/or cellular functions upon which AAV is dependent for its replication. Thus, the term captures proteins and RNAs that are required in AAV replication, including those moieties 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 such as adenovirus, herpesvirus (other than herpes simplex virus type-1) and vaccinia virus.

[0042] The term "accessory function vector" refers generally to a nucleic acid molecule that includes nucleotide sequences providing accessory functions. An accessory function vector can be transfected into a suitable host cell, wherein the vector is then capable of supporting AAV virion production in the host cell. Expressly excluded from the term are infectious viral particles as they exist in nature, such as adenovirus, herpesvirus or vaccinia virus particles. Thus, accessory function vectors can be in the form of a plasmid, phage, transposon or cosmid.

[0043] It has been demonstrated that the full-complement of adenovirus genes are not required for accessory helper functions. In particular, adenovirus mutants incapable of DNA replication and late gene synthesis have been shown to be permissive for AAV replication. Ito et al., (1970) J. Gen. Virol. 9:243; Ishibashi et al., (1971) Virology 45:317. Similarly, mutants within the E2B and E3 regions have been shown to support AAV replication, indicating that the E2B and E3 regions are probably not involved in providing accessory functions. Carter et al., (1983) Virology 126:505. However, adenoviruses defective in the E1 region, or having a deleted E4 region, are unable to support AAV replication. Thus, E1A and E4 regions are likely required for AAV replication, either directly or indirectly. Laughlin et al., (1982) J. Virol. 41:868; Janik et al., (1981) Proc. Natl. Acad. Sci. USA 78:1925; Carter et al., (1983) Virology 126:505. Other characterized Ad mutants include: E1B (Laughlin et al. (1982), *supra*; Janik et al. (1981), *supra*; Ostrove et al., (1980) Virology 104:502); E2A (Handa et al., (1975) J. Gen. Virol. 29:239; Strauss et al., (1976) J. Virol. 17:140; Myers et al., (1980) J. Virol. 35:665; Jay et al., (1981) Proc. Natl. Acad. Sci. USA 78:2927; Myers et al., (1981) J. Biol. Chem. 256:567); E2B (Carter, Adeno-Associated Virus Helped Functions, in I CRC Handbook of Parvoviruses (P. Tijssen ed., 1990)); E3

(Carter et al. (1983), *supra*); and E4 (Carter et al.(1983), *supra*; Carter (1995)). Although studies of the accessory functions provided by adenoviruses having mutations in the E1B coding region have produced conflicting results, Samulski et al., (1988) J. Virol. 62:206-210, recently reported that E1B55k is required for AAV virion production, while E1B19k is not. In addition, International Publication WO 97/17458 and Matshushita et al., (1998) Gene Therapy 5:938-945, describe accessory function vectors encoding various Ad genes. Particularly preferred accessory function vectors comprise an adenovirus VA RNA coding region, an adenovirus E4 ORF6 coding region, an adenovirus E2A 72 kD coding region, an adenovirus E1A coding region, and an adenovirus E1B region lacking an intact E1B55k coding region. Such vectors are described in International Publication No. WO 01/83797.

[0044] By "recombinant virus" is meant a virus that has been genetically altered, e.g., by the addition or insertion of a heterologous nucleic acid construct into the particle.

[0045] By "AAV virion" is meant a complete virus particle, such as a wild-type (wt) AAV virus particle (comprising a linear, single-stranded AAV nucleic acid genome associated with an AAV capsid protein coat). In this regard, single-stranded AAV nucleic acid molecules of either complementary sense, e.g., "sense" or "antisense" strands, can be packaged into any one AAV virion and both strands are equally infectious.

[0046] A "recombinant AAV virion," or "rAAV virion" is defined herein as an infectious, replication-defective virus including an AAV protein shell, encapsidating a heterologous nucleotide sequence of interest which is flanked on both sides by AAV ITRs. A rAAV virion is produced in a suitable host cell which has had an AAV vector, AAV helper functions and accessory functions introduced therein. In this manner, the host cell is rendered capable of encoding AAV polypeptides that are required for packaging the AAV vector (containing a recombinant nucleotide sequence of interest) into infectious recombinant virion particles for subsequent gene delivery.

[0047] A "caprine recombinant AAV virion" or "caprine rAAV virion" is a rAAV virion as described above that has been produced using AAV helper functions that include a gene encoding a caprine capsid protein, such as caprine VP1.

[0048] A "bovine recombinant AAV virion" or "bovine rAAV virion" is a rAAV virion as described above that has been produced using AAV helper functions that include a gene encoding a bovine capsid protein, such as a bovine VP1.

[0049] The term "transfection" is used to refer to the uptake of foreign DNA by a cell, and a cell has been "transfected" when exogenous DNA has been introduced inside the cell membrane. 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. Such techniques can be used to introduce one or more exogenous DNA moieties, such as a nucleotide integration vector and other nucleic acid molecules, into suitable host cells.

[0050] The term "host cell" denotes, for example, microorganisms, yeast cells, insect cells, and mammalian cells, that can be, or have been, used as recipients of an AAV helper construct, an AAV vector plasmid, an accessory function vector, or other transfer DNA. 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. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation.

[0051] As used herein, the term "cell line" refers to a population of cells capable of continuous or prolonged growth and division *in vitro*. Often, cell lines are clonal populations derived from a single progenitor cell. It is further known in the art that spontaneous or induced changes can occur in karyotype during storage or transfer of such clonal populations. Therefore, cells derived from the cell line referred to may not be precisely identical to the ancestral cells or cultures, and the cell line referred to includes such variants.

[0052] "homology" refers to the percent identity between two polynucleotide or two polypeptide moieties. Two DNA, or two polypeptide sequences are "substantially homologous" to each other when the sequences exhibit at least about 50%, preferably at least about 75%, more preferably at least about 80%-85%, preferably at least about 90%, and most preferably at least about 95%-98% sequence identity over a defined length of the molecules. As used herein, substantially homologous also refers to sequences showing complete identity to the specified DNA or polypeptide sequence.

[0053] In general, "identity" refers to an exact nucleotide-to-nucleotide or amino acid-to-amino acid correspondence of two polynucleotides or polypeptide sequences, respectively. Percent identity can be determined by a direct comparison of the sequence information between two molecules by aligning the sequences, counting the exact number of matches between the two aligned sequences, dividing by the length of the shorter sequence, and multiplying the result by 100. Readily available computer programs can be used to aid in the analysis, such as ALIGN, Dayhoff, M.O. in Atlas of Protein Sequence and Structure M.O. Dayhoff ed., 5 Suppl. 3:353-358, National Biomedical Research Foundation, Washington, DC, which adapts the local homology algorithm of Smith and Waterman Advances in Appl. Match. 2:482-489, 1981 for peptide analysis. Programs for determining nucleotide sequence identity are available in the Wisconsin Sequence Analysis Package, Version 8 (available from Genetics Computer Group, Madison, WI) for example, the BESTFIT, FASTA and GAP programs, which also rely on the Smith and Waterman algorithm. These programs are readily utilized with the default parameters recommended by the manufacturer and described in the Wisconsin Sequence Analysis Package referred to above. For example, percent identity of a particular nucleotide sequence to a reference sequence can be

determined using the homology algorithm of Smith and Waterman with a default scoring table and a gap penalty of six nucleotide positions.

[0054] Another method of establishing percent identity in the context of the present invention is to use the MPSRCH package of programs copyrighted by the University of Edinburgh, developed by John F. Collins and Shane S. Sturrok, and distributed by IntelliGenetics, Inc. (Mountain View, CA). From this suite of packages the Smith-Waterman algorithm can be employed where default parameters are used for the scoring table (for example, gap open penalty of 12, gap extension penalty of one, and a gap of six). From the data generated the "Match" value reflects "sequence identity." Other suitable programs for calculating the percent identity or similarity between sequences are generally known in the art, for example, another alignment program is BLAST, used with default parameters. For example, BLASTN and BLASTP can be used using the following default parameters: genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + Swiss protein + Spupdate + PIR. Details of these programs are well known in the art.

[0055] Alternatively, homology can be determined by hybridization of polynucleotides under conditions which form stable duplexes between homologous regions, followed by digestion with single-stranded-specific nuclease(s), and size determination of the digested fragments. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al., *supra*; *DNA Cloning, supra*; *Nucleic Acid Hybridization, supra*.

[0056] By the term "degenerate variant" is intended a polynucleotide containing changes in the nucleic acid sequence thereof, that encodes a polypeptide having the same amino acid sequence as the polypeptide encoded by the polynucleotide from which the degenerate variant is derived.

[0057] A "coding sequence" or a sequence which "encodes" a selected polypeptide, is a nucleic acid molecule which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A transcription termination sequence may be located 3' to the coding sequence.

[0058] The term "heterologous" as it relates to nucleic acid sequences such as coding sequences and control sequences, denotes sequences that are not normally joined together, and/or are not normally associated with a particular cell. Thus, a "heterologous" region of a nucleic acid construct or a vector is a segment of nucleic acid within or attached to another nucleic acid molecule that is not found in association with the other molecule in nature. For example, a heterologous region of a nucleic acid construct could include a coding sequence flanked by sequences not found in association with the coding sequence in nature. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Similarly, a cell transformed with a construct which is not normally present in the cell would be considered heterologous for purposes of this invention. Allelic variation or naturally occurring mutational events do not give rise to heterologous DNA, as used herein.

[0059] A "nucleic acid" sequence refers to a DNA or RNA sequence. The term captures sequences that include any of the known base analogues of DNA and RNA such as, but not limited to 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinylcytosine, pseudoisocytosine, 5-(carboxyhydroxyl-methyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethyl-aminomethyluracil, dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudo-uracil, 1-methylguanine, 1-methylinosine, 2,2-dimethyl-guanine, 2-methyladenine, 2-methylguanine, 3-methyl-cytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxy-amino-methyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine.

[0060] The term DNA "control sequences" refers collectively to promoter sequences, polyadenylation signals, transcription termination sequences, upstream regulatory domains, origins of replication, internal ribosome entry sites ("IRES"), enhancers, and the like, which collectively provide for the replication, transcription and translation of a coding sequence in a recipient cell. Not all of these control sequences need always be present so long as the selected coding sequence is capable of being replicated, transcribed and translated in an appropriate host cell.

[0061] The term "promoter" is used herein in its ordinary sense to refer to a nucleotide region comprising a DNA regulatory sequence, wherein the regulatory sequence is derived from a gene which is capable of binding RNA polymerase and initiating transcription of a downstream (3'-direction) coding sequence. Transcription promoters can include "inducible promoters" (where expression of a polynucleotide sequence operably linked to the promoter is induced by an analyte, cofactor, regulatory protein, etc.), "repressible promoters" (where expression of a polynucleotide sequence operably linked to the promoter is induced by an analyte, cofactor, regulatory protein, etc.), and "constitutive promoters".

[0062] "Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, control sequences operably linked to a coding sequence are capable of effecting the expression of the coding sequence. The control sequences need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

[0063] By "isolated" when referring to a nucleotide sequence, is meant that the indicated molecule is present in the substantial absence of other biological macromolecules of the same type. Thus, an "isolated nucleic acid molecule which encodes a particular polypeptide" refers to a nucleic acid molecule which is substantially free of other nucleic acid molecules that do not encode the subject polypeptide; however, the molecule may include some additional bases or moieties which do not deleteriously affect the basic characteristics of the composition.

[0064] For the purpose of describing the relative position of nucleotide sequences in a particular nucleic acid molecule throughout the instant application, such as when a particular nucleotide sequence is described as being situated "upstream," "downstream," "3 prime (3′)" or "5 prime (5′)" relative to another sequence, it is to be understood that it is the position of the sequences in the "sense" or "coding" strand of a DNA molecule that is being referred to as is conventional in the art.

[0065] A "functional homologue," or a "functional equivalent" of a given AAV polypeptide includes molecules derived from the native polypeptide sequence, as well as recombinantly produced or chemically synthesized polypeptides which function in a manner similar to the reference AAV molecule to achieve a desired result. Thus, a functional homologue of AAV Reap68 or Rep78 encompasses derivatives and analogues off those polypeptides—including any single or multiple amino acid additions, substitutions and/or deletions occurring internally or at the amino or carboxy termini thereof—so long as integration activity remains.

[0066] By "capable of efficient transduction" is meant that the mutated constructs of the disclosure provide for rAAV vectors or virions that retain the ability to transfect cells *in vitro* and/or *in vivo* at a level that is within 1-10% of the transfection efficiency obtained using the corresponding wild-type sequence. Preferably, the mutant retains the ability to transfect cells or tissues at a level that is within 10-100% of the corresponding wild-type sequence. The mutated sequence may even provide for a construct with enhanced ability to transfect cells and tissues. Transduction efficiency is readily determined using techniques well known in the art, including the *in vitro* transduction assay described in the Examples.

[0067] By "reduced immunoreactivity" is meant that the mutated AAV construct reacts with anti-AAV antibodies at a reduced level as compared to the corresponding wild-type AAV construct. The term "antibody" as used herein includes antibodies obtained from both polyclonal and monoclonal preparations, as well as, the following: hybrid (chimeric) antibody molecules (see, for example, Winter et al. (1991) *Nature* 349:293-299; and U.S. Patent No. 4,816,567); F(ab')₂ and F(ab) fragments; Fv molecules (non-covalent heterodimers, see, for example, Inbar et al. (1972) *Proc Natl Acad Sci USA* 69:2659-2662; and Ehrlich et al. (1980) *Biochem* 19:4091-4096); single-chain Fv molecules (sFv) (see, for example, Huston et al. (1988) *Proc Natl Acad Sci USA* 85:5879-5883); dimeric and trimeric antibody fragment constructs; minibodies (see, e.g., Pack et al. (1992) *Biochem* 31:1579-1584; Cumber et al. (1992) *J Immunology* 149B:120-126); humanized antibody molecules (see, for example, Riechmann et al. (1988) *Nature* 332:323-327; Verhoeyan et al. (1988) *Science* 239:1534-1536; and U.K. Patent Publication No. GB 2,276,169, published 21 September 1994); and, any functional fragments obtained from such molecules, wherein such fragments retain immunological binding properties of the parent antibody molecule.

[0068] The mutated constructs of the present disclosure can have reduced immunoreactivity as determined using *in vitro* and/or *in vivo* assays using any of the above types of antibodies that have been generated against the corresponding wild-type AAV construct. Preferably, the mutated AAV construct will react with such antibodies at a level at least 1.5 times lower than the corresponding wild-type construct, preferably at a level at least 2 times lower, such as at least 5-10 times lower, even at a level at least 50-100 times or at least 1000 times lower than the corresponding wild-type construct.

[0069] Preferably, the mutated AAV construct reacts at a reduced level with anti-AAV neutralizing antibodies. For example, the mutated constructs will preferably be at least 1.5 times more neutralization-resistant than the corresponding wild-type, preferably at least 2 times more neutralization-resistant, even more preferably at least 5-10 times or more, such as at least 50-100 times or more neutralization-resistant than the corresponding wild-type, as determined using standard assays, such as the *in vitro* neutralization assays described herein

[0070] The terms "subject", "individual" or "patient" are used interchangeably herein and refer to a vertebrate, preferably a mammal. Mammals include, but are not limited to, murines, rodents, simians, humans, farm animals, sport animals and pets.

[0071] The terms "effective amount" or "therapeutically effective amount" of a composition or agent, as provided herein, refer to a nontoxic but sufficient amount of the composition or agent to provide the desired response. The exact amount required will vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the condition being treated, and the particular macromolecule of interest, mode of administration, and the

like. An appropriate "effective" amount in any individual case may be determined by one of ordinary skill in the art using routine experimentation.

[0072] "Treating" or "treatment" of a disease includes: (1) preventing the disease, i.e. causing the clinical symptoms of the disease not to develop in a subject that may be exposed to or predisposed to the disease but does not yet experience or display symptoms of the disease, (2) inhibiting the disease, i.e., arresting the development of the disease or its clinical symptoms, or (3) relieving the disease, i.e., causing regression of the disease or its clinical symptoms.

2. MODES OF CARRYING OUT THE INVENTION

[0073] Before describing the present invention in detail, it is to be understood that this invention is not limited to particular formulations or process parameters 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 of the invention only, and is not intended to be limiting.

[0074] Although a number of methods and materials similar or equivalent to those described herein can be used in the practice of the present invention, the preferred materials and methods are described herein.

[0075] Central to the present invention is the discovery of novel mutant AAV sequences useful in the production of rAAV virions that display reduced immunoreactivity as compared to the corresponding wild-type virions. Furthermore, the mutants preferably retain other properties of the corresponding wild-type, such as DNA packaging, receptor binding, chromatographic purification, and the ability to transduce cells *in vitro* and *in vivo*. Preferably, such properties are within at least 1-10 % of the values measured for the corresponding AAV wild-type. More preferably such properties are within 10-100 % of the values measured for the corresponding AAV wild-type. Most preferably such properties are at least 100 % or more of the values measured for the corresponding AAV wild-type. Thus, for example, if the mutation is in an AAV-2 capsid sequence, the comparison of these attributes would be between an AAV-2 virion with the mutated capsid sequence versus an AAV-2 virion with the same components as the mutated virion except with the AAV-2 wild-type capsid protein sequence.

[0076] As explained above, the AAV mutants of the disclosure preferably display decreased immunoreactivity relative to neutralizing antibodies that may be present in the host to which the mutant virions are administered. In this way, cells and tissues of subjects that have either been naturally infected with AAV (i.e., due to previous natural infection) or artificially infected with AAV (i.e., due to previous gene therapy or nucleic acid immunization) may be more efficiently transfected with recombinant AAV virions in order to treat or prevent new or on-going disease.

[0077] A well-studied mechanism for neutralization is that a neutralizing antibody physically blocks a region on the virus required to bind to receptors that are required for infection. Previous studies with other viruses have shown that the receptors and neutralizing antibodies bind to a distinct set of amino acids and that it is possible to identify mutants at particular positions on viral capsids that affect the binding of neutralizing antibodies, but not receptors or other functions needed for viral infection. Experiments in which wild-type replicating viruses are selected to be resistant to neutralizing antibodies have shown that mutations, even in single amino acids, such as those described here, can result in significant increases in resistance to antibody neutralization.

[0078] The ability or inability of an AAV mutant virion to bind AAV antisera is partially a function of the sequence of the capsid proteins (encoded by AAV *cap* gene). Thus, the invention contemplates single, double, triple, quadruple and more amino acid changes made on the surface of the AAV virion, as well as deletions and/or insertions, in order to decrease immunoreactivity, e.g., to alter the ability of the AAV virion to bind AAV antisera. Such mutants may be assessed for resistance to neutralization and, if necessary, more drastic or multiple changes can be made.

[0079] Methods of identifying portions of the AAV virion amenable to mutation with a resulting functional rAAV virion are described in the examples below. As detailed therein, mutations to amino acids on the viral surface, such as mutations to protruding features of the capsid, including portions of the capsid known as the "spike," "cylinder" and "plateau" are preferred. Mutations are preferably to the VP2 region, more preferably to the VP3 region, and in particular, within the region of overlap between VP1, VP2 and VP3 as shown in Figure 11. Particularly preferred mutations are found within positions 80-598 of VP2 (corresponding to amino acids 217-735 of VP1 and amino acids 15-533 of VP3).

[0080] The sequence of a representative VP2 is shown in Figure 9 herein (SEQ ID NO:12). The major coat protein, VP3 spans amino acids 203-735 of VP1. The mutation comprises at least one amino acid substitution, deletion or insertion to the native protein. Representative mutations include one or more substitutions of the amino acids occurring at a position corresponding to a position of the AAV-2 VP2 capsid protein selected from the group consisting of amino acids 126, 127, 128, 130, 132, 134, 247, 248, 315, 334, 354, 357, 360, 361, 365, 372, 375, 377, 390, 393, 394, 395, 396, 407, 411, 413, 418, 437, 449, 450, 568, 569, and 571.

[0081] Generally, the naturally occurring amino acid is substituted with an amino acid that has a small side-chain and/or is uncharged and is therefore less immunogenic. Such amino acids include, without limitation, alanine, valine, glycine, serine, cysteine, proline, as well as analogs thereof, with alanine preferred. Moreover, additional mutations can be present. Representative combinations include any combination of the amino acids identified immediately above, such as but not limited to a mutation of amino acid 360 to histidine and amino acid 361 to alanine; amino acid 334 to alanine

and amino acid 449 to alanine; amino acid 334 to alanine and amino acid 568 to alanine, amino acid 568 to alanine and amino acid 571 to alanine; amino acid 334 to alanine, amino acid 449 to alanine and amino acid 568 to alanine; amino acid 571 to lysine and any of the amino acids specified above. The above combinations are merely illustrative and of course numerous other combinations are readily determined based on the information provided herein.

[0082] As described further in the examples, certain amino acids in the capsid are adjacent to the heparin-binding site. This region is termed the "dead zone" or "DZ" herein and includes amino acids G128, N131, D132, H134, N245, N246, D356, D357, H372, G375, D391, D392, E393 and E394. Amino acids in the dead zone are important for function of AAV and are thus also targets for the binding of neutralizing antibodies. As this region is important for AAV function, conservative amino acid substitutions, such as Q for H, D for E, E or N for D, and the like, are preferred in the dead zone region and result in a more functional dead zone mutant.

[0083] The various amino acid positions occurring in the capsid protein are numbered herein with reference to the AAV-2 VP2 sequence described in NCBI Accession No. AF043303 and shown in Figure 9 herein. Figure 10 shows the amino acid sequence of AAV-2 VP1. However, it is to be understood that mutations of amino acids occurring at corresponding positions in any of the AAV serotypes are encompassed by the present invention. The sequences for the capsid from various AAV serotypes isolated from multiple species are known and described in, e.g., Gao et al. (2002) Proc. Natl. Acad. Sci. URSA 99:11854-11859; Rutledge et al. (1998) J. Virol. 72:309-319; NCBI Accession Nos. NC001863; NC004828; NC001862; NC002077; NC001829; NC001729; U89790; U48704; AF369963; AF028705; AF028705; AF028704; AF513852; AF513851; AF063497; AF085716; AF43303; Y18065; AY186198; AY243026; AY243025; AY243024; AY243023; AY243022; AY243021; AY243020; AY243019; AY243018; AY243017; AY243016; AY243015; AY243014; AY243013; AY243012; AY243011; AY243010; AY243009; AY243008; AY243007; AY243006; AY243005; AY243004; AY243003; AY243002; AY243001; AY243000; AY242999; AY242998; and AY242997.

[0084] Moreover, the inventors herein have discovered a new caprine AAV, isolated from goat, termed "AAV-G1" herein. The caprine AAV VP1 sequence is highly homologous to the VP1 sequence of AAV-5, but is approximately 100 times more resistant to neutralization by existing AAV antibodies than the native AAV-5 sequence. More particularly, a 2805 bp PCR fragment of the caprine AAV described herein, encoding 603 bp of *rep*, the central intron, and all of *cap*, shows 94% homology to the corresponding AAV-5 sequence. The DNA and protein homologies for the partial *rep* are 98% and 99%, respectively. A comparison of the caprine VP1 coding sequence with a primate AAV-5 VP1 coding sequence is shown in Figures 12A-12B. The DNA for the *cap* region of the caprine AAV is 93% homologous to that of AAV-5. The amino acid sequences for the caprine VP1 versus a primate AAV-5 is shown in Figure 13. The caprine sequence encodes a VP1 protein of 726 amino acids, while AAV-5 VP1 is 724 amino acids in length. Additionally, the sequences display 94% sequence identity and 96% sequence similarity. There are 43 amino acid differences between the caprine and the primate AAV-5 VP1 sequence. With respect to the linear amino acid sequence of VP1, the distribution of the amino acid differences between AAV-5 and caprine AAV is highly polar. All of the amino acid differences occur exclusively in the C-terminal hypervariable region of VP1 in a scattered fashion. This region relative to AAV-5 and caprine includes approximately 348 amino acids from amino acid 386 to the C-terminus, numbered relative to AAV-5 VP1. The corresponding hypervariable regions in other AAV serotypes are readily identifiable and the region from a number of AAV serotypes is shown in the figures herein.

[0085] Without being bound by a particular theory, the fact that all of the amino acid differences in VP1 of AAV-5 and caprine AAV occur in regions that are probably surface exposed, implies that capsid evolution is being driven primarily by the humoral immune system of the new host and/or by adaptation to ruminant receptors.

[0086] A comparison of the VP1 sequence from caprine AAV with a number of other primate VP1 sequences, including AAV-1, AAV-2, AAV-3B, AAV-4, AAV-6, AAV-8 and AAV-5, is shown in Figures 14A-14H. The accessibility of the various amino acid positions based on the crystal structure is also shown in the figures. Moreover, the surface features of the amino acids, the location of single mutations that decrease binding and neutralization; the heparin binding sites; possible Mg²⁺ contact; the phospholipase A2 domain; as well as positions likely for base contact and DNA binding, possible phosphate and ribose contact are also shown. As can be seen in the figure, AAV-5 and caprine AAV are identical to each other at 17 positions that differ in both AAV-2 and AAV-8.

[0087] Similarly, the inventors herein have discovered a new bovine AAV, isolated from cow, termed "AAV-C1" herein. The AAV-C1 VP1 nucleotide and amino acid sequences are shown in Figures 20A and 20B, respectively. Figures 21A-21H show a comparison of the amino acid sequence of VP1 from AAV-C1 with primate AAV-1, AAV-2, AAV-3B, AAV-4, AAV-6, AAV-8, AAV-5 and caprine AAV (AAV-G1). The accessibility of the various amino acid positions based on the crystal structure is also shown in the figures. Moreover, the surface features of the amino acids, the location of single mutations that decrease binding and neutralization; the heparin binding sites; possible Mg²⁺ contact; the phospholipase A2 domain; as well as positions likely for base contact and DNA binding, possible phosphate and ribose contact are also shown.

[0088] As can be seen in the figure, VP1 from AAV-C1 shows approximately 76% identity with AAV-4. The sequence differences between AAV-4 and AAV-C1 are scattered throughout the capsid. AAV-C1 VP1 displays approximately 54% identity with AAV-5 VP1, with high homology in the Rep protein, the first 137 amino acids of AAV-5 VP1 and the non

translated region after the stop of AAV-5 VP1 (not shown). Thus, AAV-C1 appears to be a natural hybrid between AAV-5 and AAV-4. AAV-C1 also displayed approximately 58% sequence identity with VP1s from AAV-2 and AAV-8, approximately 59% sequence identity with VP1s from AAV-1 and AAV-6, and approximately 60% sequence identity with VP1 from AAV-3B.

[0089] As described in more detail in the examples, the bovine AAV is approximately 16 times more resistant to neutralization by existing AAV antibodies than the native AAV-2 sequence. Thus, the caprine and bovine sequences, and other such non-primate mammalian sequences, can be used to produce recombinant AAV virions with decreased immunoreactivity relative to primate AAV sequences, such as relative to AAV-2 and AAV-5. Additionally, regions of AAV capsids that can be mutated to provide AAV virions with reduced immunoreactivity from non-caprine and non-bovine AAV isolates and strains, such as any of the AAV serotypes, can be reasonably predicted based on the caprine and bovine AAV sequences provided herein and a comparison of these sequences and immunoreactive properties with those of other isolates and serotypes.

[0090] Based on the above discussion, and the examples provided herein, one of skill in the art can reasonably predict mutations that can be made to wild-type AAV sequences in order to generate AAV virions with decreased immunoreactivity. Amino acid changes to amino acids found on the AAV capsid surface, and especially those in the hypervariable region, are expected to provide AAV virions with decreased immunoreactivity. Moreover, based on the knowledge provided by the caprine and bovine AAV sequences, other non-primate mammalian AAVs can be identified to provide non-mutated AAV sequences for use in preparing recombinant AAV virions with decreased immunoreactivity relative to primate AAVs, such as AAV-2 and AAV-5. For example, as shown in the examples below, positions in AAV-2 mutants that correlate to neutralization resistance and that are in common between the AAV-2 mutants and caprine AAV include changes to positions 248, 354, 360, 390, 407, 413 and 449 of AAV-2.

[0091] The AAV mutants of the present disclosure can be generated by site-directed mutagenesis of the AAV cap gene region. The mutated cap region can then be cloned into a suitable helper function vector, and rAAV virions generated using the mutated helper function vector and any suitable transfection method, including the triple transfection method described herein. Mutants suitable for use with the present invention are identified by their reduced immunoreactivity, as defined above. Preferably, the mutants of the present disclosure have a reduced ability to be neutralized by anti-AAV antisera, preferably anti-AAV-2 antisera, while maintaining other biological functions such as the ability to assemble intact virions, package viral DNA, bind cellular receptors, and transduce cells.

[0092] Thus, the present invention involves the identification and use of mutated AAV sequences, as well as wild-type non-primate mammalian AAV sequences, displaying decreased immunoreactivity for incorporation into rAAV virions. Such rAAV virions can be used to deliver a "heterologous nucleic acid" (an "HNA") to a vertebrate subject, such as a mammal. As explained above, a "recombinant AAV virion" or "rAAV virion" is an infectious virus composed of an AAV protein shell (i.e., a capsid) encapsulating a "recombinant AAV (rAAV) vector," the rAAV vector comprising the HNA and one or more AAV inverted terminal repeats (ITRs). AAV vectors can be constructed using recombinant techniques that are known in the art and include one or more HNAs flanked by functional ITRs. The ITRs of the rAAV vector need not be the wild-type nucleotide sequences, and may be altered, e.g., by the insertion, deletion, or substitution of nucleotides, so long as the sequences provide for proper function, i.e., rescue, replication, and packaging of the AAV genome.

[0093] Recombinant AAV virions may be produced using a variety of techniques known in the art, including the triple transfection method (described in detail in U.S. Patent No. 6,001,650). This system involves the use of three vectors for rAAV virion production, including an AAV helper function vector, an accessory function vector, and a rAAV vector that contains the HNA. One of skill in the art will appreciate, however, that the nucleic acid sequences encoded by these vectors can be provided on two or more vectors in various combinations. As used herein, the term "vector" includes any genetic element, such as a plasmid, phage, transposon, cosmid, chromosome, artificial chromosome, virus, virion, etc., which is capable of replication when associated with the proper control elements and which can transfer gene sequences between cells. Thus, the term includes cloning and expression vehicles, as well as viral vectors.

[0094] The AAV helper function vector encodes the "AAV helper function" sequences (i.e., *rep* and *cap*), which function *in trans* for productive AAV replication and encapsidation. Preferably, the AAV helper function vector supports efficient AAV vector production without generating any detectable wild-type AAV virions (i.e., AAV virions containing functional *rep* and *cap* genes). Examples of vectors suitable for use with the present invention include pHLP19, described in U.S. Patent No. 6,001,650 and pRep6cap6 vector, described in U.S. Patent No. 6,156,303.

[0095] The accessory function vector encodes nucleotide sequences for non-AAV derived viral and/or cellular functions upon which AAV is dependent for replication (i.e., "accessory functions"). The accessory functions include those functions required for AAV replication, including, without limitation, those moieties 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 such as adenovirus, herpesvirus (other than herpes simplex virus type-1), and vaccinia virus. In a preferred embodiment, the accessory function plasmid pladen5 is used (details regarding pLaden5 are described in U.S. Patent No. 6,004,797). This plasmid provides a complete set of adenovirus accessory functions for AAV vector production, but lacks the components nec-

essary to form replication-competent adenovirus.

[0096] The rAAV vector containing the heterologous nucleic acid (HNA) may be constructed using ITRs from any of the various AAV serotypes. The HNA comprises nucleic acid sequences joined together that are otherwise not found together in nature, this concept defining the term "heterologous." To illustrate the point, an example of an HNA is a gene flanked by nucleotide sequences not found in association with that gene in nature. Another example of an HNA is a gene that itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Allelic variation or naturally occurring mutational events do not give rise to HNAs, as used herein. An HNA can comprise an anti-sense RNA molecule, a ribozyme, or a gene encoding a polypeptide.

[0097] The HNA is operably linked to a heterologous promoter (constitutive, cell-specific, or inducible) such that the HNA is capable of being expressed in the patient's target cells under appropriate or desirable conditions. Numerous examples of constitutive, cell-specific, and inducible promoters are known in the art, and one of skill could readily select a promoter for a specific intended use, e.g., the selection of the muscle-specific skeletal α -actin promoter or the muscle-specific creatine kinase promoter/enhancer for muscle cell-specific expression, the selection of the constitutive CMV promoter for strong levels of continuous or near-continuous expression, or the selection of the inducible ecdysone promoter for induced expression. Induced expression allows the skilled artisan to control the amount of protein that is synthesized. In this manner, it is possible to vary the concentration of therapeutic product. Other examples of well known inducible promoters are: steroid promoters (e.g., estrogen and androgen promoters) and metallothionein promoters.

[0098] The disclosure includes novel mutant virions comprising HNAs coding for one or more anti-sense RNA molecules, the rAAV virions preferably administered to one or more muscle cells or tissue of a mammal. Antisense RNA molecules suitable for use with the present invention in cancer anti-sense therapy or treatment of viral diseases have been described in the art. See, e.g., Han et al., (1991) Proc. Natl. Acad. Sci. USA 88:4313-4317; Uhlmann et al., (1990) Chem. Rev. 90:543-584; Helene et al., (1990) Biochim. Biophys. Acta. 1049:99-125; Agarawal et al., (1988) Proc. Natl. Acad. Sci. USA 85:7079-7083; and Heikkila et al., (1987) Nature 328:445-449. The disclosure also encompasses the delivery of ribozymes using the methods disclosed herein. For a discussion of suitable ribozymes, see, e.g., Cech et al., (1992) J. Biol. Chem. 267:17479-17482 and U.S. Pat. No. 5,225,347.

[0099] The disclosure preferably encompasses mutant rAAV virions comprising HNAs coding for one or more polypeptides, the rAAV virions preferably administered to one or more cells or tissue of a mammal. Thus, the disclosure embraces the delivery of HNAs encoding one or more peptides, polypeptides, or proteins, which are useful for the treatment or prevention of disease states in a mammalian subject. Such DNA and associated disease states include, but are not limited to: DNA encoding glucose-6-phosphatase, associated with glycogen storage deficiency type 1A; DNA encoding phosphoenolpyruvate-carboxykinase, associated with Peck deficiency; DNA encoding galactose-1 phosphate uridyl transferase, associated with galactosemia; DNA encoding phenylalanine hydroxylase, associated with phenylketonuria; DNA encoding branched chain alpha-ketoacid dehydrogenase, associated with Maple syrup urine disease; DNA encoding fumarylacetoacetate hydrolase, associated with tyrosinemia type 1; DNA encoding methylmalonyl-CoA mutase, associated with methylmalonic acidemia; DNA encoding medium chain acyl CoA dehydrogenase, associated with medium chain acetyl CoA deficiency; DNA encoding ornithine transcarbamylase, associated with ornithine transcarbamylase deficiency; DNA encoding argininosuccinic acid synthetase, associated with citrullinemia; DNA encoding low density lipoprotein receptor protein, associated with familial hypercholesterolemia; DNA encoding UDP-glucuronosyltransferase, associated with Crigler-Najjar disease; DNA encoding adenosine deaminase, associated with severe combined immunodeficiency disease; DNA encoding hypoxanthine guanine phosphoribosyl transferase, associated with Gout and Lesch-Nyan syndrome; DNA encoding biotinidase, associated with biotinidase deficiency; DNA encoding beta-glucocerebrosidase, associated with Gaucher disease; DNA encoding beta-glucuronidase, associated with Sly syndrome; DNA encoding peroxisome membrane protein 70 kDa, associated with Zellweger syndrome; DNA encoding porphobilinogen deaminase, associated with acute intermittent porphyria; DNA encoding alpha-1 antitrypsin for treatment of alpha-1 antitrypsin deficiency (emphysema); DNA encoding erythropoietin for treatment of anemia due to thalassemia or to renal failure; DNA encoding vascular endothelial growth factor, DNA, encoding angiopoietin-1, and DNA encoding fibroblast growth factor for the treatment of ischemic diseases; DNA encoding thrombomodulin and tissue factor pathway inhibitor for the treatment of occluded blood vessels as seen in, for example, atherosclerosis, thrombosis, or embolisms; DNA encoding aromatic amino acid decarboxylase (AADC), and DNA encoding tyrosine hydroxylase (TH) for the treatment of Parkinson's disease; DNA encoding the beta adrenergic receptor, DNA encoding anti-sense to, or DNA encoding a mutant form of, phospholamban, DNA encoding the sarco(endo)plasmic reticulum adenosine triphosphatase-2 (SERCA2), and DNA encoding the cardiac adenylyl cyclase for the treatment of congestive heart failure; DNA encoding a tumor suppressor gene such as p53 for the treatment of various cancers; DNA encoding a cytokine such as one of the various interleukins for the treatment of inflammatory and immune disorders and cancers; DNA encoding dystrophin, or minidystrophin and DNA encoding utrophin or miniutrophin for the treatment of muscular dystrophies; and, DNA encoding insulin for the treatment of diabetes.

[0100] The disclosure also includes novel mutant virions comprising a gene or genes coding for blood coagulation proteins, which proteins may be delivered, using the methods of the present disclosure, to the cells of a mammal having

hemophilia for the treatment of hemophilia. Thus, the invention includes: delivery of the Factor IX gene to a mammal for treatment of hemophilia, B, delivery of the Factor VIII gene to a mammal for treatment of hemophilia, A, delivery of the Factor VII gene for treatment of Factor VII deficiency, delivery of the Factor X gene for treatment of Factor X deficiency, delivery of the Factor XI gene for treatment of Factor XI deficiency, delivery of the Factor XIII gene for treatment of Factor XIII deficiency, and, delivery of the Protein C gene for treatment of Protein C deficiency. Delivery of each of the above-recited genes to the cells of a mammal is accomplished by first generating a rAAV virion comprising the gene and then administering the rAAV virion to the mammal. Thus, the disclosure includes rAAV virions comprising genes encoding any one of Factor IX, Factor VIII, Factor X, Factor VII, Factor XI, Factor XIII or Protein C.

[0101] Delivery of the recombinant virions containing one or more HNAs to a mammalian subject may be by intramuscular injection or by administration into the bloodstream of the mammalian subject. Administration into the bloodstream may be by injection into a vein, an artery, or any other vascular conduit the mutant virions into the bloodstream by way of isolated limb perfusion, a technique well known in the surgical arts, the method essentially enabling the artisan to isolate a limb from the systemic circulation prior to administration of the rAAV virions. A variant of the isolated limb perfusion technique, described in U.S. Patent No. 6,177,403, can also be employed by the skilled artisan to administer the mutant virions into the vasculature of an isolated limb to potentially enhance transduction into muscle cells or tissue. Moreover, for certain conditions, it may be desirable to deliver the mutant virions to the CNS of a subject. By "CNS" is meant all cells and tissue of the brain and spinal cord of a vertebrate. Thus, the term includes, but is not limited to, neuronal cells, glial cells, astrocytes, cerebrospinal fluid (CSF), interstitial spaces, bone, cartilage and the like. Recombinant AAV virions or cells transduced *in vitro* may be delivered directly to the CNS or brain by injection into, e.g., the ventricular region, as well as to the striatum (e.g., the caudate nucleus or putamen of the striatum), spinal cord and neuromuscular junction, or cerebellar lobule, with a needle, catheter or related device, using neurosurgical techniques known in the art, such as by stereotactic injection (see, e.g., Stein et al., J Virol 73:3424-3429, 1999; Davidson et al., PNAS 97:3428-3432, 2000; Davidson et al., Nat. Genet. 3:219-223, 1993; and Alisky and Davidson, Hum. Gene Ther. 11:2315-2329, 2000).

[0102] The dose of rAAV virions required to achieve a particular "therapeutic effect," e.g., the units of dose in vector genomes/per kilogram of body weight (vg/kg), will vary based on several factors including, but not limited to: the route of rAAV virion administration, the level of gene (or anti-sense RNA or ribozyme) expression required to achieve a therapeutic effect, the specific disease or disorder being treated, a host immune response to the rAAV virion, a host immune response to the gene (or anti-sense RNA or ribozyme) expression product, and the stability of the gene (or anti-sense RNA or ribozyme) product. One of skill in the art can readily determine a rAAV virion dose range to treat a patient having a particular disease or disorder based on the aforementioned factors, as well as other factors that are well known in the art.

[0103] Generally speaking, by "therapeutic effect" is meant a level of expression of one or more HNAs sufficient to alter a component of a disease (or disorder) toward a desired outcome or clinical endpoint, such that a patient's disease or disorder shows clinical improvement, often reflected by the amelioration of a clinical sign or symptom relating to the disease or disorder. Using hemophilia as a specific disease example, a "therapeutic effect" for hemophilia is defined herein as an increase in the blood-clotting efficiency of a mammal afflicted with hemophilia, efficiency being determined, for example, by well known endpoints or techniques such as employing assays to measure whole blood clotting time or activated prothromboplastin time. Reductions in either whole blood clotting time or activated prathratnbaplastin time are indications of an increase in blood-clotting efficiency. In severe cases of hemophilia, hemophiliacs having less than 1% of normal levels of Factor VIII or Factor IX have a whole blood clotting time of greater than 60 minutes as compared to approximately 10 minutes for non-hemophiliacs. Expression of 1% or greater of Factor VIII or Factor IX has been shown to reduce whole blood clotting time in animal models of hemophilia, so achieving a circulating Factor VIII or Factor IX plasma concentration of greater than 1% will likely achieve the desired therapeutic effect of an increase in blood-clotting efficiency.

[0104] The constructs of the present disclosure may alternatively be used to deliver an HNA to a host cell in order to elucidate its physiological or biochemical function(s). The HNA can be either an endogenous gene or heterologous. Using either an *ex vivo* or *in vivo* approach, the skilled artisan can administer the mutant virions containing one or more HNAs of unknown function to an experimental animal, express the HNA(s), and observe any subsequent functional changes. Such changes can include: protein-protein interactions, alterations in biochemical pathways, alterations in the physiological functioning of cells, tissues, organs, or organ systems, and/or the stimulation or silencing of gene expression.

[0105] Alternatively, the skilled artisan can over-express a gene of known or unknown function and examine its effects *in vivo*. Such genes can be either endogenous to the experimental animal or heterologous in nature (i.e., a transgene).

[0106] By using the methods of the present disclosure, the skilled artisan can also abolish or significantly reduce gene expression, thereby employing another means of determining gene function. One method of accomplishing this is by way of administering antisense RNA-containing rAAV virions to an experimental animal, expressing the antisense RNA molecule so that the targeted endogenous gene is "knocked out," and then observing any subsequent physiological or

biochemical changes.

[0107] The methods of the present disclosure are compatible with other well-known technologies such as transgenic mice and knockout mice and can be used to complement these technologies. One skilled in the art can readily determine combinations of known technologies with the methods of the present disclosure to obtain useful information on gene function.

[0108] Once delivered, in many instances it is not enough to simply express the HNA; instead, it is often desirable to vary the levels of HNA expression. Varying HNA expression levels, which varies the dose of the HNA expression product, is frequently useful in acquiring and/or refining functional information on the HNA. This can be accomplished, for example by incorporating a heterologous inducible promoter into the rAAV virion containing the HNA so that the HNA will be expressed only when the promoter is induced. Some inducible promoters can also provide the capability for refining HNA expression levels; that is, varying the concentration of inducer will fine-tune the concentration of HNA expression product. This is sometimes more useful than having an "on-off" system (i.e., any amount of inducer will provide the same level of HNA expression product, an "all or none" response). Numerous examples of inducible promoters are known in the art including the ecdysone promoter, steroid promoters (e.g., estrogen and androgen promoters) and metallothionein promoters.

3. EXPERIMENTAL

[0109] Insofar as the examples fall within the scope of the invention, such are to be seen as illustrative of the invention. To any other extent, the examples are provided for illustrative purposes only.

[0110] Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

EXAMPLE 1

RECOMBINANT AAV-LACZ MUTANT VIRION PREPARATION AND PROPERTIES THEREOF

[0111] Recombinant AAV-2 virions containing the β -galactosidase gene (rAAV-2 lacZ) were prepared using a triple-transfection procedure described in U.S. Patent No. 6,001,650. The complete cDNA sequence for β -gal is available under GenBank Accession No. NC 000913 REGION: complement (362455..365529).

I. Vector Construction

A. Mutant AAV Helper Function Vector

[0112] Based on the structure of AAV-2 (see, Xie et al. Proc. Natl. Acad. Sci. USA (2002) 99:10405-10410), 61 mutants were constructed by oligonucleotide-directed, site-specific mutagenesis. The entire surface of AAV is composed of 60 identical asymmetrical structural units arranged in an icosahedral shape. This has two important implications. First, any single amino acid mutation that is made will be found at 60 places on the virus all at the same position relative to other amino acids within the asymmetrical structural unit. Second, by studying a single asymmetrical structural unit one can understand the entire surface of the virus.

[0113] AAV-2 structure was determined as follows. Coordinates for the monomeric AAV-2 capsid protein (VP1 amino acids 217-735; VP2 amino acids 80-598) were obtained from the Protein Data Bank (identification number 1LP3). The structure was analyzed using Swiss PDB Viewer version 3.7, Vector NTI 3D-Mol version 8.0 (Invitrogen, Inc.), or Chime (MDL Information Systems, Inc. Multimeric structures of the AAV-2 capsid were generated using the oligomer generator program on the Virus Particle Explorer (VIPER) website, using the coordinate transformation functions of Swiss PDB viewer in conjunction with matrix coordinates in the PDB (1LP3) file, or downloaded from the protein quaternary structure database at the European Bioinformatics Institute (filename=11p3). Possible antibody binding sites on AAV-2 capsid multimers were analyzed by constructing the asymmetric structural unit of AAV-2 capsid and then manually docking an IgG structure (murine IgG2a monoclonal antibody; PDB ID number 1IGT) to that structure or to other multimeric units of the AAV-2 capsid using Swiss PDB Viewer. Distances, amino acid clashes, and contact areas between the IgG and the AAV-2 capsid could be assessed using the appropriate tools within the Swiss PDB Viewer program.

[0114] Several criteria were applied to select which amino acids out of a total of about 145 external, surface-exposed amino acids (within each of the 60 identical asymmetric structural units, see Figure 1) to mutate. Mutations were made only in external "surface-exposed" amino acids, although it is possible for amino acids under the external surface or on the internal surface to influence antibody binding. The amino acids that were mutated were those with side-chains predicted to be the most accessible to antibody binding. This included amino acids on protruding features of the capsid, known as the "spike", "cylinder", and "plateau." Such protruding features are often targets for the binding of neutralizing

antibodies. Amino acids in areas that were not wide enough to accommodate an antibody ("canyon", "dimple", center of 3-fold symmetry axis, center of 5-fold symmetry axis) were not mutated. Furthermore the amino acid side-chain was selected based on an exposed area of at least 20 Å², because decreases of 20 Å² or more in the contact area between an antigen and an antibody (out of a total contact area of approximately 600 Å² to 900 Å²) can have a measurable effect on antibody-antigen affinity and therefore on the neutralizing titer of the antibody. Amino acids selected were those with the side-chain (and not just the peptide backbone) exposed. It was assumed that if only the peptide backbone was exposed then an antibody that bound to such an amino acid may not be able to discriminate various amino acids well, since all amino acids have the same peptide backbone. Finally, relatively flat areas of protein antigens often interact with relatively flat areas of antibodies so amino acids chosen for mutation were in a relatively flat area (side of spike, top of cylinder, top of plateau). Applying all of the above criteria to the approximately 145 capsid amino acids located on the external surface of AAV-2 resulted in the selection of 72 positions that would be most likely to affect the binding of neutralizing antibodies when changed to other amino acids. The location of these amino acids is indicated in Figure 2 and listed in Tables 1, 4 and 5.

[0115] Most of the 127 mutants (at 72 positions) that were made changed single amino acids to alanine, using techniques known by those skilled in the art of molecular biology. Alanine was chosen because it has been determined that, of all mutations that could be made, alanine is the least disruptive to protein structure. Also, since alanine only has a methyl side-chain, changing most other amino acids to alanine are likely to disrupt antibody binding. That is, compared to other amino acids, alanine is less immunogenic because it lacks a side-chain that significantly contributes to antigen/antibody contact areas and hence to antigen/antibody affinity. Note that the numbering that follows is based on the AAV-2 VP2 sequence as depicted in Figure 9. A few positions were changed to an amino acid other than alanine. For example at position 356 where there already is an alanine, an arginine was inserted. Arginine is polar enough to remain on the AAV surface and large enough that it could interfere with binding of antibodies. There are five glycines that may be accessible to antibodies. Glycines are often found where a peptide chain turns and thus can be a critical component of structure. Mutation of glycines can be problematic because of the possibility that structure may be dramatically altered. Therefore each of the five glycines on the AAV-2 surface were considered on a case-by-case basis in order to decide what to change them to. G128 was changed to aspartate because glycine 128 is found in AAV-1 through 6 except for AAV-5 where position 128 is an aspartic acid. G191 was changed to serine because glycine 191 is found in AAV-1 through 6 except for AAV-5 where position 191 is a serine. G329 was changed to arginine because glycine 329 is found in AAV-1 through 6 except for AAV-4 where position 329 is an arginine. G375 was changed to proline because glycine 375 is conserved in AAV-1 through 6 and it was thought that proline might preserve a turn in the peptide chain found at that position. G449 was changed to alanine because, although it is serine or asparagine in other AAVs, it is between R448 and R451 in AAV-2, which are critical for heparin binding and transduction. Therefore position 449 was mutated to an amino acid closest in size to glycine (i.e., alanine). In some cases double mutants were isolated (S130A/N131A, N360H/S361A, S361A/N358K, S361A/S494P, S361A/R592K) in addition to the desired mutant. These were presumably a result of polymerase errors introduced during the mutagenesis, but were assayed like the other mutants.

[0116] AAV helper function vectors were constructed using pHLP19 (described in U.S. Patent No. 6,001,650), 116 mutagenic oligodeoxynucleotides, and an *in vitro* mutagenesis kit (Quik Change XL, Stratagene, San Diego, CA). Briefly, two complementary oligodeoxynucleotides that contain each desired mutant sequence and have a melting temperature between 74-83 °C. (calculated using the equation: $T_m = 81.5 + 0.41 (\%G+C) - (675/N) - \% \text{ mismatch}$, where G is guanosine, C is cytosine, N is primer length in nucleotides) were mixed separately with pHLP19. Three cycles of PCR were done using the following conditions: denaturation was performed at 95 °C. for 1 min, annealing was performed at 60 °C. for 1 min, and extension was performed at 68 °C. for 1 min. Then the two separate reactions were mixed and subjected to 18 additional cycles of PCR using the following conditions: denaturation was performed at 95 °C. for 1 min, annealing was performed at 60 °C. for 1 min, and extension was performed at 68 °C. for 15 min. The PCR products were digested with the Dpn I restriction enzyme to destroy fully methylated or hemi-methylated (i.e., non-mutant) plasmids, and then transformed into the *E. coli* strain XL-10 (Stratagene). One colony was picked from each mutagenesis reaction, 500 ng of plasmid DNA were prepared, and subjected to DNA sequencing. A subset of the mutagenic oligodeoxynucleotides were used as sequencing primers to confirm the sequences of mutants. The entire capsid gene was sequenced in each case. Most mutants could be isolated in this manner. If a mutant was not isolated by the first round of DNA sequencing, 1-3 more colonies were picked and 500 ng of plasmid DNA was prepared and subjected to DNA sequencing.

B. pLadeno5 Accessory Function Vector

[0117] The accessory function vector pLadeno5 was constructed as follows. DNA fragments encoding the E2a, E4, and VA RNA regions isolated from purified adenovirus serotype-2 DNA (obtained from Gibco/BRL) were ligated into a plasmid called pAmpscript. The pAmpscript plasmid was assembled as follows. Oligonucleotide-directed mutagenesis was used to eliminate a 623-bp region including the polylinker and alpha complementation expression cassette from pBSII s/k+ (obtained from Stratagene), and replaced with an EcoRV site. The sequence of the mutagenic oligo used on

the oligonucleotide-directed mutagenesis was 5'-CCGCTACAGGGCGCGATATCAGCTCACTCAA-3' (SEQ ID NO:1).
[0118] A polylinker (containing the following restriction sites: Bam HI; KpnI; SrfI; XbaI; ClaI; Bst1 107I; Sall; PmeI; and NdeI) was synthesized and inserted into the EcoRV site created above such that the BamHI side of the linker was proximal to the f1 origin in the modified plasmid to provide the pAmpscript plasmid. The sequence of the polylinker was

5'-

GGATCCGGTACCGCCCGGGCTCTAGAATCGATGTATACGTCGACGTTTAA

ACCATATG-3' (SEQ ID NO:2).

[0119] DNA fragments comprising the adenovirus serotype-2 E2a and VA RNA sequences were cloned directly into pAmpscript. In particular, a 5962-bp SrfI-KpnI(partial) fragment containing the E2a region was cloned between the SrfI and KpnI sites of pAmpscript. The 5962-bp fragment comprises base pairs 21,606-27,568 of the adenovirus serotype-2 genome. The complete sequence of the adenovirus serotype-2 genome is accessible under GenBank No. 9626158.

[0120] The DNA comprising the adenovirus serotype-2 E4 sequences was modified before it was inserted into the pAmpscript polylinker. Specifically, PCR mutagenesis was used to replace the E4 proximal, adenoviral terminal repeat with a SrfI site. The location of this SrfI site is equivalent to base pairs 35,836-35,844 of the adenovirus serotype-2 genome. The sequences of the oligonucleotides used in the mutagenesis were: 5'-AGAGGCCCGGGCGTTTTAGGGCG-GAGTAACTTGC-3' (SEQ ID NO:3) and 5'-ACATACCCGCAGCGTAGAGAC-3' (SEQ ID NO:4). A 3,192 bp E4 fragment, produced by cleaving the above-described modified E4 gene with SrfI and SpeI, was ligated between the SrfI and XbaI sites of pAmpscript which already contained the E2a and VA RNA sequences to result in the pLadeno5 plasmid. The 3,192-bp fragment is equivalent to base pairs 32,644-35,836 of the adenovirus, serotype-2 genome.

C. rAAV-2 hF.IX vector

[0121] The rAAV-2 hF.IX vector is an 11,442-bp plasmid containing the cytomegalovirus (CMV) immediate early promoter, exon 1 of hF.IX, a 1.4-kb fragment of hF.IX intron 1, exons 2-8 of h.FIK, 227 bp of h.FIX 3' UTR, and the SV40 late polyadenylation sequence between the two AAV-2 inverted terminal repeats (see, U.S. Patent No. 6,093,392). The 1.4-kb fragment of hF.IX intron 1 consists of the 5' end of intron 1 up to nucleotide 1098 and the sequence from nucleotide 5882 extending to the junction with exon 2. The CMV immediate early promoter and the SV40 late polyadenylation signal sequences can be obtained from the published sequence of pCMV-Script®, which is available from the Stratagene catalog, Stratagene, La Jolla, CA.

D. rAAV-2 lac Z vector

Construction of the recombinant AAV plasmid pVmLacZ

[0122]

1. A 4311 bp Xba I DNA fragment was excised from pSUB201 which contains AAV rep/cap sequences. The Xba I ends were reannealed with a 10 bp Not I synthetic oligonucleotide (5'-AGCGGCCGCT-3') (SEQ ID NO:5) to give a plasmid intermediate pUC/ITR-Not I that has both AAV ITR's (inverted terminal repeats) separated by 116 bp of residual AAV sequence and Not I linker DNA.

2. A 1319 bp Not I DNA fragment was excised from p1.1c containing CMV promoter and hGH intron sequences. This DNA sequence was inserted into the Not I site of pUC/ITR-Not I, to give the intermediate pSUB201N.

3. A 1668 bp Pvu II (5131-1493) ITR bound CMV expression cassette was excised from pSUB201N and inserted at the Pvu II site (position 12) of pWee.1a, to give the plasmid intermediate pWee.1b. The excision of the 1668bp PvuII fragment from pSUB201N removed 15 bp from the outside of each ITR, in the "A" palindromic region.

4. A 4737 bp Not I/Eco RV "AAVrep/cap" DNA sequence was excised from pGN1909 and the ends were rendered blunt by filling in the 3' recessed ends using Klenow DNA polymerase. Asc I linkers were ligated to both ends, followed by cloning this "pGN1909/AscI" DNA fragment into the backbone of pWee.1b at an Asc I site (2703), to give the intermediate pWee1909 (8188bp). This plasmid has the ITR-bound CMV expression cassette with an AAV rep/cap gene backbone.

5. A 3246 bp Sma I/Dra I LacZ gene was excised from pCMV-beta and Asc I linkers were ligated to the blunt-ended fragment. This LacZ/Asc I fragment was cloned into p1.1c between Bss HII sites, to give p1.1cADHLacZ, that has the LacZ gene driven by the CMV promoter.

6. A 4387 bp Not I DNA fragment was excised from p1.1cADHLacZ, that has

the LacZ gene driven by the CMV promoter. This fragment was inserted between the Not I sites of pWee1909, after removing a 1314b p "CMV promoter/hGH intron" expression cassette. The resulting construct, pW1909ADHLacZ, has the β -galactosidase gene under the control of the CMV promoter and bounded by ITRs. The backbone of the plasmid carries the "rep" and "cap" genes providing AAV helper functions and the β -lactamase (ampicillin) gene confers antibiotic resistance.

7. A 4772 bp Sse I DNA fragment containing a "CMV/LacZ" cassette was excised from pW1909ADHLacZ and inserted into the Sse I site of pUC19, to give Pre-pVLacZ. This construct still contains approximately 50 bp of remnant 5' and 3' pSUB201 sequences internal to each ITR.

8. The remnant pSUB201 sequences were removed by excising a 2912 bp Msc I "pUC/ Δ ITR" DNA fragment from Pre-pVLacZ, that also removes approximately 35 bp of the "D" region of each ITR. A synthetic linker "145NA/NB" (5'-CCAACTCCATCACTAGGGGTTCTGCGGCC-3') (SEQ ID NO:6) containing an Msc I restriction site, the ITR "D" region and a Not I site was used to clone in a 4384 bp Not I fragment from pW1909ADHLacZ, that has the "CMV/LacZ" expression cassette. The resulting plasmid pVLacZ, is has the β -galactosidase gene under the control of an alcohol dehydrogenase enhancer sequence and the CMV promoter, all bounded by AAV ITRs.

9. pVLacZ was further modified by removing LacZ elements and polylinker sequence outside of the ITR bound LacZ expression cassette as follows. A 534 bp Ehe I/Afl III LacZ/polylinker sequence was excised from pUC 119, the ends were blunted using Klenow DNA polymerase and the plasmid was ligated to a Sse I linker (5'-CCTGCAGG-3') (SEQ ID NO:7), to produce pUC119/SseI. The "AAVLacZ" DNA sequence was removed from pVLacZ by cutting out a 4666 bp Sse I fragment. This SseI fragment was cloned into the Sse I site of pUC119/SseI to generate pVmLacZ. pVmLacZ has the CMV promoter/ADH enhancer/ β -galactosidase gene bounded by AAV ITRs in a pUC119-derived backbone that confers ampicillin resistance and has a high copy number origin of replication.

II. Triple Transfection Procedure

[0123] The various mutated AAV helper function vectors (described above), the accessory function vector pLadeno5 (described in U.S. Patent No. 6,004,797), and the rAAV2-lacZ vector, pVmLacZ (described above) were used to produce recombinant virions.

[0124] Briefly, human embryonic kidney cells type 293 (American Type Culture Collection, catalog number CRL-1573) were seeded in 10 cm tissue culture-treated sterile dishes at a density of 3×10^6 cells per dish in 10 mL of cell culture medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and incubated in a humidified environment at 37° C in 5% CO₂. After overnight incubation, 293 cells were approximately eighty-percent confluent. The 293 cells were then transfected with DNA by the calcium phosphate precipitate method, a transfection method well known in the art. 10 μ g of each vector (mutated pHLP19, pLadeno5, and pVm lacZ.) were added to a 3-mL sterile, polystyrene snap cap tube using sterile pipette tips. 1.0 mL of 300 mM CaCl₂ (JRH grade) was added to each tube and mixed by pipetting up and down. An equal volume of 2X HBS (274 mM NaCl, 10 mM KCl, 42 mM HEPES, 1.4 mM Na₂PO₄, 12 mM dextrose, pH 7.05, JRH grade) was added with a 2-mL pipette, and the solution was pipetted up and down three times. The DNA mixture was immediately added to the 293 cells, one drop at a time, evenly throughout the dish. The cells were then incubated in a humidified environment at 37° C in 5% CO₂ for six hours. A granular precipitate was visible in the transfected cell cultures. After six hours, the DNA mixture was removed from the cells, which were then provided with fresh cell culture medium without fetal calf serum and incubated for an additional 72 hours.

[0125] After 72 hours, the cells were lysed by 3 cycles of freezing on solid carbon dioxide and thawing in a 37 °C. water bath. Such freeze-thaw lysates of the transfected cells were characterized with respect to total capsid synthesis (by Western blotting), DNA packaging (by Q-PCR), heparin binding, *in vitro* transduction (on HeLa or HepG2 cells plus adenovirus-2 or etoposide), and neutralization by antibodies.

III. Properties of the mutant virions,

A. Capsid Synthesis Assay

[0126] Mutations in proteins can render them unstable and more susceptible than normal to degradation by proteases. In order to determine the level of capsids made by the mutants described herein, western blotting of crude lysates was performed. One microliter of each crude lysate was denatured by incubation in 20 mM Tris, pH 6.8, 0.1% SDS at 80 °C. for 5 minutes. Proteins were fractionated by SDS-PAGE using 10% polyacrylamide gels (Invitrogen, Inc., Carlsbad, CA) and then detected by western blotting as follows. The proteins were electrophoretically blotted (Xcell II blot module, Invitrogen, Carlsbad, CA) onto nylon membranes (Hybond-P, Amersham Biosciences, Piscataway, N.J.). The membranes were probed with an anti-AAV antibody (monoclonal clone B1, Maine Biotechnology Services, Inc. Portland, ME) at a dilution of 1:20 and then with a sheep anti-mouse antibody coupled to horseradish peroxidase (Amersham Bio-

sciences, Piscataway, N.J.) at a dilution of 1:12000. The B1 antibody-binding proteins were detected using the ECL Plus western blotting detection system (Amersham Biosciences, Piscataway, N.J.). The membranes were exposed to x-ray film Biomax MS, Kodak, Rochester, NY) for 1-5 minutes and the signals were quantified using an Alphamager 3300 (Alpha Innotech Corp., San Leandro, CA)

B. DNA packaging assay.

[0127] Quantitative polymerase chain reaction (Q-PCR) was used to assess DNA packaging by AAV-2 virions with mutant capsids. In this procedure the crude lysate was digested with DNase I prior to PCR amplification to remove any plasmid (used in transfection) that might result in a false positive signal. The crude lysates were diluted 100 fold (5 μ l crude lysate plus 495 μ l buffer) in 10 mM Tris, pH 8.0, 10 μ g/ml yeast tRNA. An aliquot of the dilution (10 μ l) was digested with 10 units DNase I (Roche Molecular Biochemicals, Indianapolis, IN) in 25 mM Tris, pH 8.0, 1 mM MgCl₂ at 37 °C. for 60 minutes in a final volume of 50 μ l. The DNase I was inactivated by heating at 95 °C. for 30 minutes. One microliter (20 μ g) of proteinase K (Roche Molecular Biochemicals, Indianapolis, IN) was added and incubated 55 °C. for 30 minutes. The Proteinase K was inactivated by heating at 95 °C. for 20 minutes. At this point, the sample was diluted in 10 mM Tris, pH 8.0, 10 μ g/ml yeast tRNA if necessary. Ten microliters of DNase 1 and proteinase K-treated sample was added to 40 μ l Q-PCR master mix, which consisted of:

4 μ l H₂O

5 μ l 9 μ M lac Z primer #LZ-1883F (5'-TGCCACTCGCTTTAATGAT-3', (SEQ ID NO:8) Operon, Inc., Alameda, CA)

5 μ l 9 μ M lac Z primer #LZ-1948R (5'-TCGCCGCACATCTGAACTT-3', (SEQ ID NO:9) Operon, Inc., Alameda, CA)

1 μ l 10 μ M lacZ probe # LZ-1906T (5'-6FAM-AGCCTCCAGTACAGCGCGGCTGA-TAMRA-3', (SEQ ID NO:10) Applied Biosystems, Inc. Foster City, CA)

25 μ l TaqMan Universal PCR Master Mix (Applied Biosystems, Inc. Foster City, CA)

[0128] Q-PCR amplification was done using an Applied Biosystems model 7000 Sequence Detection System according to the following program. There were two initial incubations at 50 °C. for 2 minutes and 95 °C. for 10 minutes to activate Taq polymerase and denature the DNA template, respectively. Then the DNA was amplified by incubation at 95 °C. for 15 sec, then 60 °C. for 60 seconds for 40 cycles. A standard curve was constructed using 4-fold dilutions of linearized pVm lac Z ranging from a copy number of 61 to 1,000,000. The copy number of packaged rAAV-lacZ genomes in each sample was calculated from the C_t values obtained from the Q-PCR using the Applied Biosystems Prism 7000 Sequence Detection System version 1.0 software.

C. Heparin-binding Assay

[0129] Heparin binding of viruses in crude lysates was performed as follows. Twenty microliters of crude cell lysate containing AAV-2 virions with wild-type or mutant capsids were mixed with 25 μ l of a 50% slurry of heparin beads. The heparin beads (Ceramic Hyper-DM Hydrogel-Heparin, Biosepra, Cergy-Saint-Christophe, France) were 80 μ m in diameter and had 1000 Å pores to allow AAV (which is ~300 Å in diameter) access to the heparin. The beads were washed thoroughly in phosphate-buffered saline prior to use. The beads and virions were incubated at 37 °C. for 60 minutes. The beads were pelleted. The supernatant containing unbound virions was saved. The beads were washed 2 times with 500 μ l PBS. The supernatants were combined and unbound capsid proteins were precipitated with trichloroacetic acid at a final concentration of 10%. Precipitated proteins were denatured by incubation in 20 mM Tris, pH 6.8, 0.1% SDS at 80 °C. for 5 minutes. Virions bound to heparin beads were released by incubation of the beads in 20 mM Tris, pH 6.8, 0.1% SDS at 80 °C. for 5 minutes. All protein samples prepared in this manner were fractionated by molecular weight by SDS-PAGE using 10% polyacrylamide gels (Invitrogen, Inc., Carlsbad, CA) and then detected by western blotting as follows. The proteins were electrophoretically blotted onto nylon membranes (Hybond-P, Amersham Biosciences, Piscataway, N.J.). The membranes were probed with an anti-AAV antibody (monoclonal clone B1, Maine Biotechnology Services, Inc. Portland, ME) at a dilution of 1:20 and then with a sheep anti-mouse antibody coupled to horseradish peroxidase (Amersham Biosciences, Piscataway, N.J.) at a dilution of 1:12000. The B1 antibody-binding proteins were detected using the ECL Plus western blotting detection system (Amersham Biosciences, Piscataway, N.J.). The membranes were exposed to x-ray film Biomax MS, Kodak, Rochester, NY) for 1-5 minutes and the signals were quantitated using an Alphamager 3300 (Alpha Innotech Corp., San Leandro, CA)

D. *In vitro* transduction assay.

[0130] HeLa cells (American Type Culture Collection, catalog # CCL-2) were plated in 24-well dishes at 5e4 cells per well. Cells were grown for 24 hr in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% fetal

bovine serum (Gibco) and penicillin-streptomycin (Gibco) at 37 °C. Ten-fold dilutions of crude lysates containing the control wild type and mutant viruses were made in DME/10 % FBS. The virus dilutions were added to the cells along with wild type adenovirus-5 (American Type Culture Collection, catalog # VR-5). The amount of adenovirus used was 0.1 µl per well, which was titrated previously and shown to maximally stimulate rAAV-2 lac Z transduction of HeLa cells. After 24 hours at 37 °C the cells were fixed using 2 % formaldehyde and 0.2 % glutaraldehyde and stained for β-galactosidase activity using 1 mg/ml (2.5 mM) 5-bromo-4-chloro-3-indolyl β-D galactopyranoside in PBS, 2 mM MgCl₂, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, pH 7.2. After another 24 hours, the number of blue cells in four random microscopic fields were counted and averaged for each well. Instead of using HeLa cells and adenovirus-5, HepG2 cells and 20 µM etoposide could also be used and similar results were obtained.

E. Antibody and serum neutralization assays.

[0131] Hep G2 cells (American Type Culture Collection, catalog # HB-8065) were plated in 24-well dishes at 1.5e5 cells per well. Cells were grown for 24 hr in Minimum Essential Medium (Eagle's) (KMEM) (ATCC) supplemented with 10 % fetal bovine serum and penicillin-streptomycin at 37 °C. Two-fold dilutions of the A20 antibody (Maine Biotechnology, Portland, ME) were made using PBS. Wild-type and mutant virus was diluted by mixing 1 microliter of crude lysate of the viral preparation with 15 microliters of KMEM/0.1 % Bovine Serum Albumin (BSA). Samples of KMEM/0.1 % BSA and PBS were included as a negative controls. A total of 16 µL of A20 dilution was mixed with 16 µL of virus and incubated at 37 °C for one hour. Ten microliters of virus/A20 mixture was added to each of three wells of cells. After one hour incubation at 37 °C, etoposide (20 mM stock solution in dimethyl sulfoxide, Calbiochem) was added to each well at a final concentration of 20 µM. After 24 hours the cells were fixed using 2 % formaldehyde and 0.2 % glutaraldehyde and stained for β-galactosidase activity using 1 mg/ml (2.5 mM) 5-bromo-4-chloro-3-indolyl β-D galactopyranoside in PBS, 2 mM MgCl₂, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, pH 7.2. After another 24 hours, the number of blue cells in four random microscopic fields were counted and averaged for each well. The neutralizing titer of an antibody is defined as the dilution of antibody at which there is a 50 % reduction in the number of viral transduction events (i. e., blue cells) compared to transduction in the absence of antibody.

[0132] Neutralization of mutants by human sera collected from hemophiliacs or to purified human IgG from > 10,000 donors (Panglobulin, ZLB Bioplasma AG, Berne, Switzerland) was assayed in the same manner. For purified human IgG, a concentration of 10 mg/ml was considered to be equivalent to undiluted sera since the normal concentration of IgG in human sera varies from 5-13 mg/ml.

F. ELISAs.

(a) A20 ELISA:

[0133] An ELISA kit (American Research Products, Belmont, MA) that uses a monoclonal antibody (A20) to capture and detect AAV-2 was used to quantitate particle numbers. The kit was used according to the manufacturer's instructions. Optical density was measured in a Spectramax 340PC plate reader (Molecular Devices, Sunnyvale, CA) at 450 nm wavelength. The concentration of virus needed to result in a half maximal optical density reading was calculated and used to compare the results from different samples.

(b) IgG/A20 ELISA:

[0134] Microtiter plates (96-well EIA/RIA flat bottom, high-binding polystyrene, Costar, Corning, NY) were coated using 100 µl (10 µg) Panglobulin in 0.1 M sodium bicarbonate buffer, pH 9.2 for 16 hours at 20 °C. Plates were blocked with 200 µl PBS, 1 % BSA, 0.05 % Tween-20 for 1 hour at 20 °C. Increasing amounts of CsCl gradient-purified native or mutant AAV-2 ranging from 3.0⁸ to 1.0¹⁰ vector genomes per well were added and incubated for 16 hours at 20 °C. Unbound virus was washed off using 3-200 µl aliquots of PBS, 0.1 % Tween-20 buffer. A20-biotin from the AAV-2 ELISA kit was diluted 1:50, 100 µl was added per well, and incubated for 1 hours at 37 °C. Unbound A20-biotin was washed off using 3 200 µl aliquots of PBS, 0.1 % Tween-20 buffer. Then streptavidin coupled to horseradish peroxidase was diluted 1:20 and incubated for 1 hours at 37 °C. Unbound streptavidin-HRP was washed off using 3 200 µl aliquots of PBS, 0.1 % Tween-20 buffer. Horseradish peroxidase substrates (Immunopure TMB substrate kit Pierce, Rockford, IL) were added and incubated for 15 min at 20 °C. The reaction was stopped with 100 µl 2M sulfuric acid and optical density was measured in a Spectramax 340PC plate reader (Molecular Devices, Sunnyvale, CA) at 450 nm wavelength. The concentration of virus needed to result in a half maximal optical density reading was calculated and used to compare the results from different samples.

(c) IgG ELISA:

[0135] Microtiter plates (96-well EIA/RIA flat bottom, high-binding polystyrene, Costar, Coming, NY) were coated with increasing amounts of CsCl gradient-purified native or mutant AAVs-2 ranging from 3.0^8 to 1.0^{10} vector genomes per well for 16 hours at 20 °C. in 0.1 M sodium bicarbonate buffer, pH 9.2 for 16 hours at 20 °C. Plates were blocked with 200 μ l PBS, 1 % BSA, 0.05 % Tween-20 for 1 hour at 20 °C. Unbound virus was washed off using 3-200 μ l aliquots of PBS, 0.1 % Tween-20 buffer. Panglobulin was added and incubated for 1 hour at 37 °C. Unbound Panglobulin was washed off using 3-200 μ l aliquots of PBS, 0.1 % Tween-20 buffer. Then donkey, anti-human IgG coupled to horseradish peroxidase (Amersham Biosciences, Piscataway, NJ) was added and incubated for 1 hours at 37 °C. Unbound secondary antibody was washed off using 3-200 μ l aliquots of PBS, 0.1 % Tween-20 buffer. Horseradish peroxidase substrates (Immunopure TMB substrate kit Pierce, Rockford, IL) were added and incubated for 15 min at 20 °C. The reaction was stopped with 100 μ l of 2M sulfuric acid and optical density was measured in a Spectramax 340PC plate reader (Molecular Devices, Sunnyvale, CA) at 450 nm wavelength. The concentration of virus needed to result in a half maximal optical density reading was calculated and used to compare the results from different samples.

[0136] The DNA packaging, heparin-binding, and transduction properties of mutants described here are summarized in Table 1. The antibody neutralization properties of some of the mutants described here are summarized in Tables 2 and 3.

Table 1. Properties of AAV-2 capsid mutants.

Mutant ¹	Capsid synthesis ²	DNA packaging ³	Heparin binding ⁴	Transduction ⁵
wild type	100	100	>95	100
Q126A	65	67	>95	55
Q126A/S127L	78	4	>95	0.02
S127A	68	98	>95	53
G128D	100	674	>95	0.02
Δ 128ins1	77	777	>95	0.02
S130A/N131A	55	nt	>95	0.02
N131A	67	563	>95	0.005
D132A	75	23	>95	0.04
H134A	44	540	>95	2
Q188A	55	16	>95	0.36
D190A	60	51	>95	95
G191S	108	18	>95	22
T193A	38	7	>95	6
S247A	18	83	>95	24
Q248A	60	374	>95	280
S315A	101	122	>95	232
T317A	101	111	>95	208
T318A	100	132	>95	224
Q320A	97	89	>95	68
R322A	100	560	>95	106
G329R	43	21	>95	0.24
S331A	168	80	>95	158
D332A	85	474	>95	8
R334A	169	601	>95	79
D335A	136	127	>95	38
T354A	132	301	>95	93
S355A	69	353	>95	38
S355T	110	183	>95	88
A356R	85	18	25	13
D357A	39	166	>95	4
N359A	24	365	>95	89
N360A	8	246	>95	33
N360H/S361A	145	472	>95	38

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(continued)

Mutant ¹	Capsid synthesis ²	DNA packaging ³	Heparin binding ⁴	Transduction ⁵
S361A	81	608	>95	89
5 S361A/N358K	59	nt	>95	0.45
S361A/S494P	87	nt	90	0.02
S361A/R592K	108	nt	90	180
E362A	149	56	>95	12
10 W365A	195	60	>95	4
T366A	151	8	>95	0.01
G375P	221	82	50	0.01
D377A	211	80	>95	20
K390A	155	267	>95	189
15 D392A	98	48	>95	0.01
E393A	54	81	>95	2
E394A	29	108	>95	22
K395A	34	2046	>95	14
F396A	178	nt	>95	148
20 K407A	220	112	>9-5	32
E411A	90	513	>95	20
T413A	233	34	>95	252
E418A	264	74	>95	37
25 K419A	81	806	>95	160
E437A	239	94	>95	24
Q438A	28	101	>95	92
G449A	104	106	>95	196
N450A	217	144	>95	207
30 Q452A	313	533	>95	473
N568A	439	412	>95	536
K569A	831	333	>95	20
V571A	98	251	>95	142

¹ Mutants are named as follows: The first letter is the amino acid in wild type AAV-2 capsid, the number is the position in capsid that was mutated (numbered according to the AAV-2 VP2 sequence), and the last letter is the mutant amino acid. Δ128ins1 has amino acid 128 deleted and the sequence DASNDNLSSQSD inserted in its place.

² As determined by western blotting of crude lysates. Expressed as a percentage of wild type capsid synthesis.

³ DNase-resistant, vector-specific DNA, quantified by Q-PCR and expressed as a percentage of wild type, which was normalized to 100 %. Average of 2 experiments, each done in triplicate. nt, not tested.

⁴ Heparin-binding, expressed as a percentage of wild type. Single determinations except for wild type, which is an average of three determinations, normalized to 100%.

⁵ Transduction on human 293 cells expressed as a percentage of wild type. Average of 2 experiments.

Table 2. Antibody neutralization properties of AAV-2 capsid mutants.

		Transduction blue cells blue cells			% Neut.	
Fold neutralization	Serum ¹ Mutant resistant	(% of wt)	(- serum)	(+ serum)		
HA2	wild type	100	13275	3	99.98	1.0
	R334A	114	15102	146	99.04	5.2
	42.7					
	N450A	89	11802	14	99.88	1.0
	wild type	100	25960	8	99.97	1.0
	E394A	6	1593	6	99.64	

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(continued)

		Transduction blue cells blue cells						
Fold neutralization Serum ¹ Mutant resistant		(% of wt)	(- serum)	(+ serum)	% Neut.			
5		11.2						
		T413A	21	5505	15	99.73	8.5	
		N360H/S361A	41	10691	7	99.94	2.0	
10	HA151	wild type	100	11965	16	99.87	2.0	
		R334A	185	22125	459	97.93	1.0	
		15.8						
		E394A	16	1947	14	99.27	5.6	
15		V571A	73	8732	39	99.56	3.4	
		G449A	218	26137	121	99.54	3.5	
20		N568A	122	14632	36	99.75	1.9	
		N450A	95	11387	53	99.54	3.5	
25		wild type	100	15989	13	99.92	1.0	
		E411A	18	2876	13	99.54	5.7	
30		N360H/S361A	100	15989	21	99.87	1.6	
	HA165	wild type	100	22833	14	99.94	1.0	
		N360A	16	3717	9	99.75	4.0	
35		R334A	74	16872	162	99.04		
		15.3						
		E394A	11	2566	2	99.91	1.4	
40		N568A	102	23246	30	99.87	2.1	
		N450A	64	14514	26	99.82	2.9	
		N360H/S361A	49	9558	8	99.92	1.3	

¹ Mutants were rapidly screened by comparing the number of transduced cells resulting from infection of FiepG2 cells by rAAV-2 lac Z with mutant or wild type capsids in the presence or absence of a monoclonal (A20) antibody at a dilution of 1:80 or human polyclonal serum at a dilution of 1:100.

Table 3. Antibody titration properties of 4 antibodies against AAV-2 capsid mutants.

		Fold decrease in neutralizing titer ¹			
Antibody ² :		A20	151	165	HA2
Mutant ³					
	wild type	1.0	1.0	1.0	1.0
55	Q126A	2.5	NR	NR	NR
	S127A	57.0	NR	NR	NR
	S247A	2.8	NR	NR	NR

(continued)

		Fold decrease in neutralizing titer ¹			
Antibody ² :		A20	151	165	HA2
5	Mutant³				
	Q248A	5.7	NR	NR	NR
	R334A	NR	3.6	2.4	2.0
	N360H/S361A	NR	2.2	1.2	1.3
10	E394A	NR	2.1	1.2	1.9
	N450A	NR	1.7	1.6	1.3
Predicted multiplicative resistance:					
		2415	29	6	11

¹ Titers were determined by using 2-fold dilutions of monoclonal antibody and fitting the data to a four-parameter logistic curve using Sigma Plot graphing software. Values reported in the table are the fold decrease in titer of the mutant relative to wild type capsid. NR, not resistant to neutralization by indicated antibody.

² A20 is a protein A-purified anti-AAV-2 mouse monoclonal antibody. Sera 151, 165, and HA2 are 3 unpurified human sera.

³ Mutants are named as follows: The first letter is the amino acid in wild type AAV-2 capsid, the number is the position in capsid that was mutated (numbered according to the AAV-2 VP2 sequence), and the last letter is the mutant amino acid. Δ128ins1 has amino acid 128 deleted and the sequence DASNDNLSSQSD (SEQ ID NO:11) inserted in its place.

[0137] As can be seen, by changing single amino acids on the surface of AAV-2 32 mutants out of 61 were identified that had nearly normal properties with respect to capsid synthesis, DNA packaging, heparin binding, and transduction of cells *in vitro*. Ten mutants were more resistant to neutralization by antibodies.

[0138] The mutants made capsid protein at a level between 5-fold lower to 8-fold higher than wild type. They packaged DNA at a level between 25-fold lower to 20-fold higher than wild type. With regard to transduction, 28 of the mutants transduced at least 50 % as well as wild type, 16 transduced 10-50 % of wild type, 6 transduced 1-10 % of wild type, and 11 transduced less than 1 % of wild type (Table 1). There were no significant differences in transduction of human cervical carcinoma-derived HeLa cells or human liver-derived Hep G2 cells, or when either adenovirus or etoposide was used to enhance transduction. Several mutants reproducibly had up to 5-fold more transducing activity than wild type (Table 1).

[0139] Most of the mutants with <1% transduction activity were clustered in a single area, on one side of the (proposed) heparin-binding site (Table 1, compare Figure 4 with Figure 5). Without being bound by a particular theory, the mutations cover an area that may be a protein-binding site. The mutant that was most defective for transduction was N131A. A function for N131 has not been described, but it is conserved in 40 out of 42 known AAV subtypes.

[0140] Four mutations affected heparin binding more noticeably than the others (A356R, G375A, S361A/S494P, S361A/R592K). Each of these is near R347, R350, K390, R448 and R451, which have been previously identified as amino acids that are important for heparin binding (Figure 5).

[0141] Forty five of the mutants (Q126A, S127A, D190A, G191S, S247A, Q248A, S315A, T317A, T318A, Q320A, R322A, S331A, D332A, R334A, D335A, T354A, S355A, S355T, A356R, D357A, N359A, N360A, N360H/S361A, S361A, S361A/R592K, E362A, D377A, K390A, E393A, E394A, K395A, F396A, K407A, E411A, T413A, E418A, K419A, E437A, Q438A, G449A, N450A, Q452A, N568A, K569A, V571A) with more than approximately 10 % of the transduction activity of wild-type AAV-2 capsid were screened for neutralization by the murine A20 monoclonal antibody. Four mutants (Q126A, S127A, S247A, Q248A) were significantly more resistant to neutralization by A20 than was AAV2 with a wild type capsid (see Table 3). The titer of these mutants (Q126A, S127A, S247A, Q248A) was 1:203, 1:9, 1:180 and 1:89, respectively (Figure 8), which is 2.5, 57, 2.8, and 5.7-fold greater than the neutralizing titer of the A20 monoclonal antibody against wild type AAV-2 capsid (1:509). These 4 mutants are located immediately adjacent to each other on the surface of the AAV-2 capsid (Figure 6).

[0142] Three (Q126A, S127A, Q248A) of the four mutations that reduce neutralization by A20 were essentially normal with regard to capsid synthesis, DNA, packaging, heparin binding, and transduction. Capsid synthesis and transduction by mutant S247A was 4- to 5-fold less than wild-type AAV-2 capsid. Thus it is possible to have a virus that is normal in several important properties but has increased resistance to antibody neutralization.

[0143] The mutant rAAV virions Q126A, S127A, S247A, Q248A yielded an unexpected 2.5- to 57-fold resistance to neutralizing antibody while maintaining transduction efficiency in 2 different human cell lines (HeLa and HepG2). These four amino acids are immediately adjacent to each other on the surface of AAV-2 (Figure 6). Furthermore, they are in

an area that had been previously implicated in binding the A20 antibody, based on peptide competition and insertional mutagenesis experiments. Based on these observations it is possible the A20 antibody blocks one or more functions necessary for AAV-2 to transduce cells. In a previous study it has been shown that A20 does not block binding of AAV-2 to heparin (Wobus et al (2000) J. Virol. 74:9281-93). The results reported here support this data since mutations that affect heparin binding are located far from mutations that affect A20 binding. Although A20 does not block heparin binding, it does prevent AAV-2 from entering cells. It is possible that A20 does not interfere with binding to a "docking receptor" such as heparin, but instead interferes with binding of AAV-2 to an "entry receptor". Two proteins have been described that are required for AAV-2 transduction which may be entry receptors: the basic fibroblast growth factor receptor (bFGFR) and $\alpha_v\beta_5$ integrin. The areas on AAV-2 that these receptors may bind have not been identified. It is possible $\alpha_v\beta_5$ integrin, bFGFR, or both may bind to the localized area described herein that has a high concentration of mutants that are significantly defective in transduction (<1% of normal). Note that the area that is most defective for transduction is located adjacent to the mutants that affect A20 binding.

[0144] The same 45 mutants (Q126A, S127A, D190A, G191S, S247A, Q248A, S315A, T317A, T318A, Q320A, R322A, S331A, D332A, R334A, D335A, T354A, S355A, S355T, A356R, D357A, N359A, N360A, N360H/S361A, S361A, S361A/R592K, E362A, D377A, K390A, E393A, E394A, K395A, F396A, K407A, E411A, T413A, E418A, K419A, E437A, Q438A, G449A, N450A, Q452A, N568A, K569A, V571A) with more than approximately 10 % of the transduction activity of wild type AAV-2 capsid were screened for neutralization by 3 human neutralizing antisera. Four mutants (R334A, N360H/S361A, E394A, N450A) were identified in an initial screen that were more resistant to neutralization by all three human antisera, than was AAV2 with a wild- type capsid (see Table 2). The titer of antisera when tested on these mutants ranged from 1.3 to 3.6-fold greater than the neutralizing titer of the three human antisera against wild type AAV-2 capsid (Table 3). Six other mutants (N360A, E411A, T413A, G449A, N568A, V571A) had increased levels of resistance to neutralization by 1 or 2 of the 3 sera tested (Table 2).

[0145] The location of the mutations that confer antibody neutralization resistance is informative. First, mutants that confer resistance to a mouse monoclonal antibody are located immediately adjacent to each other on the surface of the AAV-2 capsid whereas those that confer resistance to human antisera are spread over a larger area (Figure 7). This suggests the human antisera are polyclonal, which is not surprising. Second, both sets of mutants are located on the plateau and spike but not on the cylinder, even though the cylinder would be readily accessible to antibody binding. Third, mutations that affect neutralization are near areas important for AAV function. Several mutants that affect neutralization by human antisera (at positions 360, 394, 449, 450) are located within 2 amino acids of the heparin binding site, which is likely to be a functionally important target for binding by neutralizing antibodies. Other mutants (at positions 126, 127, 247, 248, 334, 568, 571) are located at the periphery of the large region on the plateau (dead zone) that contains most of the mutants that had <10% of wild type transduction activity (Figure 4). Like the heparin-binding site, this area presumably has an important function and is likely to be a functionally important target for binding by neutralizing antibodies.

[0146] When multiple mutations that confer resistance to antibody neutralization are combined the cumulative resistance to antibody neutralization is often multiplicative, especially when the individual mutations result in low levels of resistance. Therefore, it is likely that if the mutants described here are combined into one capsid, those capsids could be 5-fold to over 1000-fold more resistant to neutralization compared to a wild-type capsid (Table 3). Dilutions of A20 greater than 1:1000 neutralized <3% of wild-type AAV-2. Thus a mutant with a combination of the 4 single amino acids that provide some resistance to neutralization by A20 could be almost completely resistant to neutralization even by undiluted A20 antisera.

[0147] Although mutants with <10% wild type transduction activity may also be resistant to antibody neutralization they were not tested because the neutralization assay, as described here, works best when used to assay mutants that have >~10% of wild-type transduction activity (Figure 3). This is because it is desirable to be able to detect neutralization over a wide range of antibody concentrations so that a titer can be accurately calculated. However, mutants with <10% wild-type transduction activity could still be tested for their ability to bind neutralizing antibody using a modification of the assay described here in which a transduction defective mutant would be used as a competitor. For example a wild-type "reporter" rAAV-2 lacZ virus could be mixed with a transduction defective "competitor" AAV-2 that lacks any genome ("empty virus") or with an AAV-2 virus that has packaged another gene (e.g., green fluorescent protein). If a "competitor" AAV-2 protects a reporter AAV-2 from neutralization then the "competitor" capsid should be able to bind neutralizing antibody and thus would not be resistant to neutralization. If a "competitor" AAV-2 does not protect a reporter AAV-2 from neutralization then the "competitor" capsid may not be able to bind neutralizing antibody and thus could be resistant to neutralization as long as it was shown to make a normal amount of capsid. In this way even mutants that are transduction defective but resistant to antibody neutralization could be identified. In order to make such mutants useful as vehicles for delivering genes in the presence of neutralizing antibodies, it would be desirable to find an amino acid substitution other than alanine that would restore normal transducing activity, but still retain decreased susceptibility to neutralization.

[0148] 66 more mutants were made and tested using the protocols described above. The DNA packaging, heparin-binding, and transduction properties of the additional mutants are summarized in Table 4.

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Table 4. Properties of Additional AAV-2 capsid mutants.

	Mutant	Capsid synthesis ²	DNA packaging	Heparin binding	Transduction
5	G128A	+	207	>95%	1.5
	S130A	+	172	>95%	92
	S130T	+	232	>95%	1164
	N131Q	+	113	>95%	0.01
10	D132E	+	202	>95%	4
	D132N	+	188	>95%	75
	N133A	+	187	>95%	418
	H134F	+	180	>95%	0.2
	H134Q	+	340	>95%	17
15	H134T	+	102	>95%	0.4
	N245A	+	145	>95%	1.8
	G246A	+	353	>95%	0.6
	R350K	+	52	>95%	16
20	D357E	+	222	>95%	427
	D357N	+	157	>95%	28
	D357Q	+	204	>95%	1.6
	N360H	+	129	>95%	37
	N360K	+	59	>95%	0.06
25	W365F	+	253	>95%	6
	T366S	+	251	>95%	18
	H372F	+	130	>95%	4.1
	H372K	+	154	>95%	72
30	H372N	+	221	>95%	122
	H372Q	+	248	>95%	73
	G375A	+	55	>95%	2.4
	D391A	+	140	>95%	1.21
	D392E	+	158	>95%	15
35	D392I	+	411	>95%	0.5
	D392N	+	236	>95%	0.2
	D392V	+	247	>95%	0.001
	E393D	+	218	>95%	80
40	E393K	+	123	>95%	0.02
	E393Q	+	92	>95%	1.2
	E394K	+	190	>95%	6.0
	E411K	+	28	>95%	4.6
	T413K	+	196	>95%	57
45	R448A	+	3255	< 1%	0.3
	R448K	+	768	>95%	80
	G449K	+	270	>95%	3.1
	N450K	+	281	>95%	0.7
50	R451A	+	2971	< 1%	0.07
	R451K	+	10	>95%	133
	N568K	+	488	>95%	16
	V571K	+	614	>95%	40
	R334A/N360K	+	380	>95%	0.6
55	R334A/G449A	+	87	>95%	91
	R334A/N450A	+	738	>95%	238
	R334A/N568A	+	150	>95%	147

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(continued)

	Mutant	Capsid synthesis ²	DNA packaging	Heparin binding	Transduction
5	N360K/N450A	+	166	>95%	0.2
	E411A/T413A	+	548	>95%	74
	G449A/N450A	+	94	>95%	111
	G449A/N568A	+	102	>95%	105
	G449K/ N568K	+	284	>95%	0.02
10	N568A/V571A	+	139	>95%	59
	R334A/N360K/ E394A	+	38	>95%	0.8
	R334A/N360K/ E394A ins2 ¹	+	21	>95%	0.001
	R334A/N360K/ G449K	+	320	>95%	0.01
15	R334A/G449A/ N568A	+	746	>95%	424
	R334A/G449K/ N568K	+	50	>95%	2.0
	R347C/G449A/ N450A	+	102	50%	0.02
	R334A/N360K/ N450A	+	26	>95%	0.3
	R334A/N360K/ E394A/N450A	+	445	>95%	0.9
20	R334A/N360K/ G449K/N568K	+	26	>95%	0.001
	E411A/T413A/ G449A/N450A	+	372	>95%	74
	E411A/T413A/ G449A/N450A/ N568A/ V571A	+	437	>95%	14
25	R334A/N360K/ E394A/E411A/ T413A/G449A	+	152	>95%	0.006
	N450A/N568A/ V571A				

¹ ins2 is an insertion of the sequence HKDDEAKFFPQ after VP2 amino acid 399.

² += within 10-fold of wild type.

[0149] As shown in Table 4, several mutants were obtained with increased transduction as compared to wild-type capsids. For example, mutants S130T, N133A, D357E, H372N, R451K, G449A/N450A, R334A/N450A, R334A/G449A/N568A, R334A/N568A, G449A/N568A displayed increased transduction. Mutant S130T was the best transducer, with approximately 11 times over wild-type levels. This was remarkable because the only difference between S (serine) and T (threonine) is a CH₂ group. Also as seen in Table 4, combination mutants usually transduced at the same level as that of the single mutant with the lowest level of transduction.

[0150] Certain amino acids in the capsid overlap the heparin-binding site. This region is termed the "dead zone" or "DZ" herein. Mutations in the dead zone can result in capsids that still bind one of the AAV-2 receptors (e.g., heparin) but do not transduce cells. Amino acid substitutions were made in dead zone amino acids and these substitutions were compared to substitution of the same amino acid with alanine. Results are shown in Table 5.

Table 5. Effect of non-alanine substitutions in dead zone.

	Dead zone position	Substitution	Transduction (% of wild type)
45	G128	A	1.5
		D	0.02
	N131	A	0.005
		Q	0.01
	D132	A	0.04
50		E	4
		N	75
	H134	A	2
55		F	0.2
		Q	17
		T	0.4

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(continued)

	Dead zone position	Substitution	Transduction (% of wild type)
5	D357	A	4
		E	427
		N	128
		Q	1.6
10	H372	A	0.008 ^a
		F	4
		K	72
		N	122
15		Q	73
	G375	A	2.4
		P	0.01
20	D392	A	0.01
		E	15
		I	0.5
		N	0.2
		V	0.001
25	E393	A	2
		D	80
		K	0.2
		Q	1.2
30	^a Data from Opie, S.R., et al., J. Virology 77,6995-7006, (2003)		

[0151] As shown above, the more conservative the substitution the more functional the dead zone mutant was. For example Q was a good substitute for H. D was a good substitute for E. E or N were good substitutes for D. It was not a surprise that glycine, which has several unique properties was difficult to substitute.

[0152] The heparin binding properties of mutant G375P (transduction 0.01 % of wild-type) and G375A (transduction 2.4% of wild-type) were compared. Mutant G375P bound heparin at 50% and G375A at 95%. Position 375 might be required for both dead zone and heparin binding site function. Substitution of glycine with alanine in the G375A mutant results in a phenotype that is the same as other dead zone mutants - it binds heparin normally but displays <10% of normal transduction. However, substitution of glycine with proline in the G375P mutant results in a phenotype more similar to a mutant defective in heparin binding (such as R347C/ G449A/ N450A). Without being bound by a particular theory, the differences in structure between glycine, alanine, and proline imply that the side chain of glycine may be required for dead zone function, since substitution with alanine reduces transduction. The amine group may be required for heparin binding since substitution with proline, which does not have an amine group, affects heparin binding. Alternatively proline substitution may disrupt the structure of the heparin binding site from a distance.. There were three mutants (R448A, R451A, R347C/G449A/N450A) that didn't bind heparin, but these were in positions previously known to be required for heparin binding (347, 448,451).

[0153] Neutralization activity of several of these mutants by murine monoclonal antibody (A20) and also by a purified, pooled human IgG was determined. The pooled human IgG preparation was used as it is well characterized, commercially available, highly purified, and it is believed to represent nearly all antigen specificities that would be found in the United States which was the source of blood used to purify the IgG. Results are shown in Table 6.

Table 6. Neutralization by purified, pooled human IgG and murine monoclonal antibody A20

Mutant	Fold decrease in neutralizing titer ¹	Fold decrease in A20 titer
WT	1.0	
S127A	2.2 *	
G128A	4.1 *	

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(continued)

	Mutant	Fold decrease in neutralizing titer ¹	Fold decrease in A20 titer
	S130A	1.4	
5	S130T	1.8	
	D132N	3.8 *	
	N133A	0.9	
	H134Q	1.5	
10	R334A	2.2 *	
	T354A	2.9 *	
	D357E	1.7	
	D357N	1.8	
	N360H/S361A	2.1 *	
15	W365A	10.4 *	0.5
	H372K	1.1	
	G375P	1.9	
	D377A	1.9	
20	K390A	2.3 *	
	E394A	1.5	
	E394K	2.3 *	0.9
	K395A	4.9 *	0.9
	F396A	1.6	
25	K407A	3.3 *	1.6
	E411A		2.7*
	T413K	2.6 *	
	E418A	1.5	
	E437A	2.0 *	0.8*
30	Q438A	1.3	
	R448K	1.0	
	G449A	2.5 *	
	N450A	1.6	
35	Q452A	1.3	
	N568A	2.0 *	
	K569A	4.0 *	1.7
	V571A	3.9 *	1.4
	V571K	1.0	217*
40	R334A/ G449A	3.9 *	
	R334A/ N568A	2.4 *	
	G449A/ N568A	1.7	
	N568A/ V571A	2.5 *	
45	R334A/ G449A/N568A	3.0 *	
	E411A/ T413A/G449A/ N450A	1.0	
	E411A/ T413A/ G449A/ N450A/N568A/V571A	1.3	

¹ *=statistically significant, p<0.05. Titers were determined by doing 2-fold dilutions of IgG. The data was plotted using Sigma Plot software and the reciprocal of the dilution at which 50% neutralization occurred is defined as the titer.

[0154] As shown in the table, 21 mutants (S127A, G128A, D132N, R334A, T354A, N360H/S361A, W365A, K390A, E394K, K395A, K407A, T413K, E437A, G449A, N568A, K569A, V571A, R334A/ G449A, R334A/ N568A, N568A/ V571A, R334A/G449A/ N568A) were from 2-10 fold more resistant to neutralization by a large pool of human IgG compared to native AAV-2 capsid. As would be expected, some of the mutants that were resistant to neutralization by pooled human IgG were also resistant to neutralization by individual human sera (e.g., R334A, N360H/S361A, G449A, N568A, V571A). Without being bound by a particular theory, epitopes that contain those amino acids may bind antibody with high affinity or at high frequency. However, some mutants resistant to neutralization by pooled human IgG were not identified as

resistant to individual sera, possibly because epitopes that contain those amino acids are more rarely found in the human population. In addition, some mutants were resistant to neutralization by individual sera but not to pooled human IgG (e.g., E394A, N450A). In these cases it is possible the antibodies that bind to epitopes that contain these amino acids are low affinity or low abundance such that mutations that affect their binding are not detectable in the context of a large complex mixture of IgG.

[0155] As can be seen in Figure 7, these mutations are scattered at various locations across the surface of AAV-2. The size of the area they cover is 2-3 times the size of an average epitope, implying there may be at least 2-3 epitopes involved in neutralization by the sum total of all human IgGs.

[0156] Combinations of single, neutralization resistance mutants sometimes resulted in a slightly higher degree of neutralization resistance compared to the single mutants that comprised a multiple mutant. However the degree of the effect clearly is not multiplicative for these mutants at these levels of neutralization resistance.

[0157] Two more mutants resistant to neutralization by the murine monoclonal antibody A20 were also identified: E411A which is 2.7-fold resistant and V571K which is 217-fold resistant to neutralization by A20. The V571K mutant provides evidence for a concept termed by the present inventors as "lysine scanning". Rather than removing part of an antibody binding site by changing an amino acid with a large side chain to one with a smaller side chain such as alanine, the concept of lysine scanning is to substitute an amino acid that has a small side chain (e.g., V571) with lysine which has a large side chain. Rather than removing part of an antibody binding site as might be the case for alanine substitutions, the aim of lysine scanning is to insert larger amino acids that could sterically interfere with antibody binding. Lysine was chosen since it is commonly found on the surface of AAV-2 and thus likely to be an accepted substitution. However, other large amino acids such as arginine, tryptophan, phenylalanine, tyrosine, or glutamine may also result in a similar effect without compromising biological activity. Note that while V571A is not resistant to neutralization by the murine A20 antibody, V571K is 217 fold more resistant to neutralization by A20 than is native V571 AAV-2 capsid.

[0158] V571K is located on the plateau, immediately adjacent to the four other mutants identified as resistant to A20 neutralization (Q126A, S127A, S247A, Q248A; Table 3). However E411A is located on the spike, albeit close enough to Q126A, S127A, S247A, Q248A and V571K to be within the same epitope. Inclusion of E411 in the A20 epitope evidences that A20 may bind to both the plateau and the spike, i.e. across the canyon. Molecular modeling suggests that one of AAV-2 receptors, the basic FGF receptor (PDB ID: 1FQ9), could fit very well in the AAV-2 canyon (in a manner and location remarkably similar to the way the transferrin receptor is thought to bind to canine parvovirus). If the basic FGF receptor binds to the AAV-2 canyon, then binding of A20 across the canyon would block binding of the basic FGF receptor and explain the observation that A20 neutralizes AAV-2 by blocking entry, a step in transduction that the basic FGF receptor is likely to mediate.

[0159] The plateau and spike area may bind antibodies that neutralize other AAVs by preventing receptor binding. For example AAV-5 has been shown to require the PDGF receptor for entry into cells (Di Pasquale et al., Nature Medicine (2003) 9:1306-1312). Although the structure of the PDGF receptor is not known, it is homologous in amino acid sequence to the basic FGF receptor. For example, both are composed of similar repetitive Ig-like sequence domains and thus would be expected to have similar 3-dimensional structures. Thus, it is possible that the PDGF receptor may bind to the AAV-5 canyon.

[0160] V571A, but not V571K is resistant to neutralization by pooled human IgG. Conversely V571K, but not V571A is resistant to neutralization by murine monoclonal A20. It is possible that antibodies in the human IgG pool bind directly to V571. Substitution of the valine side chain for the smaller alanine side chain may result in less binding by human IgG. The lysine side chain may still provide enough hydrophobic contacts to allow binding to occur, but not be so large as to prevent binding. A20 may not bind directly to V571 (explaining the absence of an effect of the V571A mutant on binding or neutralization by A20). However A20 clearly binds in the vicinity of V571. It is possible that V571K indirectly interferes with A20 binding, for example by steric interference.

[0161] An IgG ELISA was also done. There are many potential mechanisms of neutralization, especially *in vivo*. Binding of an IgG to AAV in a region that is not required for the function of AAV could still lead to reduction of the ability of AAV to deliver genes. For example, the primary function of macrophages is to bind foreign organisms that are bound to antibodies. When an antibody-bound organism is bound to a macrophage (via Fc receptors) the foreign organism is engulfed and destroyed. Another potential route that antibodies could use in order to neutralize AAV is by cross-linking. Antibodies are bivalent and AAV would likely have 60 antibody binding sites per epitope (and possibly multiple epitopes). Thus, as is well documented in the scientific literature, at certain antibody and virus concentrations, a cross-linked network of AAVs and antibodies can form. Such immune complexes can become so large that they precipitate or become lodged in the vasculature prior to reaching a target organ. For this reason, antibodies that bind AAV *in vivo*, on areas of AAV that are not functionally significant, can result in reduced transduction as much as antibodies that do bind to functionally significant areas. Results are shown in Table 7.

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Table 7. IgG ELISA

Mutant		Fold decrease in binding of human IgG	Fold decrease in binding of murine A20
	Wild type	1	1
5	S130A	1	1
	S130T	1	1
	D132N	1	1
	H134Q	1	1
	G246A	1	1
10	R334A	1	1
	D357E	1	1
	N360H	1	1
	H372K	1	1
15	H372Q	1	1
	E393D	1	1
	T413K	1	1
	G449A	1	1
	N568K	1	1
20	N568A	1	1
	V571K	10	10
	E411A, T413A	1	1
	N568A, N571A	1	1
25	E411A, T413A, G449A, N450A	1	1
	R334A, G449A, N568A	1	1
30	R334A, G449A	1	1
	R334A, N568A	1	1
	G449A, N568A	1	1

[0162] As shown in Table 7, one mutant (V571K) was identified that bound both A20 and a pool of human IgG 10 times worse than native AAV-2. In the all-A20 ELISA binding of mutant V571K was reduced 10-fold. In an all-human IgG ELISA binding of mutant V571K was reduced 10-fold. When an A20/IgG sandwich ELISA format was used, binding of mutant V571K was reduced 100-fold. Position (571) is immediately adjacent to positions 126, 127, 247 and 248 on the surface of the AAV-2 capsid. Positions 126, 127, 247 and 248 were identified as important for neutralization by the mouse monoclonal antibody A20. Therefore this region may be antigenic in both mice and humans.

[0163] To summarize, several mutations to the external surface of AAV-2 capsid that reduced neutralization by antibodies, but had minimal effects on biological properties were identified. In particular, 127 mutations were made at 72 positions (55 % of surface area) deemed most likely to be accessible to antibody binding based on manual docking of IgG and AAV-2 structures. Single alanine substitutions (57), single non-alanine substitutions (41), multiple mutations (27), and insertions (2) were made. All mutants made capsid proteins and packaged DNA at levels within 10-fold of wild type. All mutants bound heparin as well as wild-type, except for six which were close to or within the heparin binding site. 42 of 98 single mutants transduced at least as well as wild-type. Several mutants had increased transducing activity. One, an S to T mutant, had 11-fold greater transducing activity than wild type. Combination (up or down) mutants usually transduced at the same level as that of the single mutants with the lowest level of transduction.

[0164] 13 of 15 single alanine substitution mutants with <10 % transduction activity were adjacent to each other in an area (10% of surface) that overlaps the heparin-binding site. Although these "dead zone (DZ)" mutants had from 0.001%-10% of normal transduction activity, all of them bound heparin as efficiently as wild-type. Transduction by DZ mutants could be increased, and in three cases restored to wild-type levels, by making conservative substitutions.

[0165] Five mutants had reduced binding to a mouse monoclonal antibody (A20) in an ELISA and were 2.5-217 fold more resistant to neutralization by A20 in vitro. These 5 mutants were adjacent to each other and to the DZ. A total of 21 single mutants were 2-10 fold resistant to neutralization by three human sera or by a large pool of purified human IgG (IVIG, Panglobulin) compared to wild-type. Different sets of mutations conferred resistance to different human sera. The location of these mutations was widespread. The size of the area they covered suggested human sera neutralize

AAV-2 by binding at least two epitopes. Three mutants were resistant to all sera tested, but combinations of these three mutants did not increase resistance to neutralization by IVIG. One (V to K) mutant was identified that bound IVIG 10-fold worse than wild-type in an all-IVIG ELISA. However, this mutant was not resistant to IVIG neutralization.

[0166] In summary, mutations in the "dead zone" affect transduction, but not heparin binding. Mutations around the DZ can increase transduction or decrease binding of antibodies. The DZ is very acidic (6 acidic, 0 basic amino acids). Without being bound by a particular theory, it may be a binding site for a basic protein, such as bFGF or the bFGF receptor. Since the dead zone is adjacent to the heparin binding site on AAV-2 it may be that if a protein binds to the dead zone, then that protein may also bind heparin. Both bFGF and the bFGF receptor bind heparin.

EXAMPLE 2

FACTOR IX EXPRESSION IN MICE USING MUTANT AAV-hF.IX

[0167] rAAV-F.IX is prepared using the rAAV-2 hF.IX vector and the methods described above. Freeze-thaw lysates of the transfected cells are precipitated, rAAV virions are purified by two cycles of isopycnic centrifugation; and fractions containing rAAV virions are pooled, dialysed, and concentrated. The concentrated virions are formulated, sterile filtered (0.22 μ M) and aseptically filled into glass vials. Vector genomes are quantified by the "Real Time Quantitative Polymerase Chain Reaction" method (Real Time Quantitative PCR. Heid C.A., Stevens J., Livak K.J., and Williams P.M. 1996. Genome Research 6:986-994. Cold Spring Harbor Laboratory Press).

[0168] Female mice 4-6 weeks old are injected with mutant rAAV-hF.IX virions. Mice are anesthetized with an intraperitoneal injection of ketamine (70 mg/kg) and xylazine (10 mg/kg), and a 1 cm longitudinal incision is made in the lower extremity. Mutant recombinant AAV-hF.IX (2×10^{11} viral vector genomes/kg in HEPES-Buffered-Saline, pH 7.8) virions is injected into the tibialis anterior (25 μ L) and the quadriceps muscle (50 μ L) of each leg using a Hamilton syringe. Incisions are closed with 4-0 Vicryl suture. Blood samples are collected at seven-day intervals from the retro-orbital plexus in microhematocrit capillary tubes and plasma assayed for hF.IX by ELISA. Human F.IX antigen in mouse plasma is assessed by ELISA as described by Walter et al. (Proc Natl Acad Sci USA (1996) 3:3056-3061). The ELISA does not cross-react with mouse F.IX. All samples are assessed in duplicate. Protein extracts obtained from injected mouse muscle are prepared by maceration of muscle in PBS containing leupeptin (0.5 mg/mL) followed by sonication. Cell debris is removed by microcentrifugation, and 1:10 dilutions of the protein extracts are assayed for hF.IX in the ELISA. Circulating plasma concentrations of HF.IX is measured by ELISA at various time points post-IM injection (e.g., zero, three, seven, and eleven weeks).

EXAMPLES 3

HEMOPHILIA B TREATMENT IN DOGS WITH MUTANT AAV-cF.IX

[0169] A colony of dogs having severe *hemophilia* B comprising males that are hemizygous and females that are homozygous for a point mutation in the catalytic domain of the canine factor IX (cF.IX) gene, is used to test the efficacy of cF.IX delivered by mutant rAAV virions (rAAV-cF.IX). The severe hemophilic dogs lack plasma cF.IX, which results in an increase in whole blood clotting time (WBCT) to >60 minutes (normal dogs have a WBCT between 6-8 minutes), and an increase in activated partial thromboplastin time (aPTT) to 50-80 seconds (normal dogs have an aPTT between 13-18 seconds). These dogs experience recurrent spontaneous hemorrhages. Typically, significant bleeding episodes are successfully managed by the single intravenous infusion of 10 mL/kg of normal canine plasma; occasionally, repeat infusions are required to control bleeding.

[0170] Under general anesthesia, hemophilia B dogs are injected intramuscularly with rAAV1-cF.IX virions at a dose of 1×10^{12} vg/kg. The animals are not given normal canine plasma during the procedure.

[0171] Whole blood clotting time is assessed for cF.IX in plasma. Activated partial thromboplastin time is measured. A coagulation inhibitor screen is also performed. Plasma obtained from a treated hemophilic dog and from a normal dog is mixed in equal volumes and is incubated for 2 hours at 37° C. The inhibitor screen is scored as positive if the aPTT clotting time is 3 seconds longer than that of the controls (normal dog plasma incubated with imidazole buffer and pro-treatment hemophilic dog plasma incubated with normal dog plasma). Neutralizing antibody titer against AAV vector is assessed.

EXAMPLE 4HEMOPHILIA B TREATMENT IN HUMAN WITH MUTANT AAV-hF.IXA. Muscle Delivery

[0172] On Day 0 of the protocol patients are infused with hF.IX concentrate to bring factor levels up to ~100%, and, under ultrasound guidance, mutant rAAV-h.FIX virions are injected directly into 10-12 sites in the *vastus lateralis* of either or both anterior thighs. Injectate volume at each site is 250-500 μ L, and sites are at least 2 cm apart. Local anesthesia to the skin is provided by ethyl chloride or eutectic mixture of local anesthetics. To facilitate subsequent muscle biopsy, the skin overlying several injection sites is tattooed and the injection coordinates recorded by ultrasound. Patients are observed in the hospital for 24 h after injection; routine isolation precautions will be observed during this period to minimize any risk of horizontal transmission of virions. Patients are discharged and seen daily in outpatient clinic daily for three days after discharge, then weekly at the home hemophilia center for the next eight weeks, then twice monthly up to five months, then monthly for the remainder of the year, then annually in follow-up. Circulating plasma levels of hF.IX are quantified using ELISA as described above.

B. Liver Delivery

[0173] Using the standard Seldinger technique, the common femoral artery is cannulated with an angiographic introducer sheath. The patient is then heparinized by IV injection of 100U/kg of heparin. A pigtail catheter is then advanced into the aorta and an abdominal aortogram is performed. Following delineation of the celiac and hepatic arterial anatomy, the proper HA is selected using a standard selective angiography catheter (Simmons, Sos-Omni, Cobra or similar catheters). Prior to insertion into the patient, all catheters are flushed with normal saline. Selective arteriogram is then performed using a non-ionic contrast material (Omnipaque or Visipaque). The catheter is removed over a 0.035 wire (Bentsen, angled Glide, or similar wire). A 6F Guide-sheath (or guide catheter) is then advanced over the wire into the common HA. The wire is then exchanged for a 0.018 wire (FlexT, Microvena Nitenol, or similar wire) and a 6X2 Savvy balloon is advanced over the wire into the proper HA distal to the gastroduodenal artery. The wire is then removed, the catheter tip position confirmed by hand injection of contrast into the balloon catheter, and the lumen flushed with 15 ml of heparinized normal saline (NS) to fully clear the contrast. Prior to infusion of the AAV-hFIX, the balloon is inflated to 2 atm to occlude the flow lumen of the HA. AAV-hFIX, at a dose of 8×10^{10} - 2×10^{12} , is brought to a final volume of approximately less than or equal to 40 ml (depending on dose and weight of patient) and is then infused over 10-12 minutes using an automatic volumetric infusion pump. Three milliliters (ml) of normal saline (NS) are then infused (at the same rate as the AAV-hFIX), to clear the void volume of the catheter. The balloon remains inflated for 2 minutes at which time the balloon is deflated and the catheter removed. A diagnostic arteriogram of the femoral puncture site is then performed in the ipsilateral anterior oblique projection. The puncture site is closed by standard methods, e.g., utilizing a percutaneous closure device using either a 6 F Closer (Perclose Inc., Menlo Park, CA) or a 6 F Angioseal, or by manual compression applied for 15 to 30 minutes at the site of catheter removal.

EXAMPLE 5ISOLATION AND CHARACTERIZATION OF A NEW CAPRINE AAVA. Cell Culture and Virus Isolation

[0174] Ovine adenovirus preparations with evidence of parvovirus contamination were isolated from caprine ileum as follows. Tissue was homogenized in Eagle's MEM medium containing Earles salts (PH 7.2) and gentomycin. The homogenate was clarified by low speed centrifugation ($1,500 \times g$) for 20 minutes and filter-sterilized through a 0.45μ m device. Supernatant (500 μ l) was inoculated onto a 25cm² flask containing primary cultures of fetal lamb kidney cells at passage 3 and incubated with fetal bovine serum (USA) and lactalbumin hydrolysate (USA) at 37° C in humid, 5% CO₂ incubator for one week. Cells were trypsinized, split, and incubated again as described above and finally assayed for typical adenoviral cytopathic effect (CPE). Flasks showing CPE were frozen at -70° C, thawed and layered onto other cell types. These flasks were later incubated and tested for CPE.

[0175] Other cell types used included non-immortalized (passage 8) ovine fetal turbinate cells derived from fetal ovine tissue and Maden Darby bovine kidney cells, maintained by long-term passage (used at passage 160). Porcine trypsin (USA) was used in all tissue culture processes and no human cell cultures or products were used.

B. Viral DNA Isolation and AAV Sequence Identification and Comparison

[0176] Four preparations from different cell cultures and passages were processed individually for DNA extraction. Virus-containing supernatant was treated with proteinase K (200 μ g) in digestion buffer (10 mM Tris-HCl (PH 8.0), 10 mM EDTA (PH 8.0) and 0.5% SDS) and incubated at 37° C for 1 hour. Following phenol chloroform extraction and ethanol precipitation the viral DNA was resuspended in TE.

[0177] The DNA content of each preparation was determined by PicoGreen DNA quantitation (Molecular Probes, Eugene, OR) and the preparations were diluted to 20 ng/ μ l to standardize DNA concentration for subsequent PCR assays.

Oligonucleotide primers

[0178] Oligonucleotide primers were selected on the basis of sequence alignments from segments that were highly conserved among known AAVs. The forward primer 1 (GTGCCCTTCTACGGCTGCGTCAACTGGACCAATGA-GAACTTTCC) (SEQ ID NO:23), was complementary to the helicase domain and the reverse primer 2 (GGAATCG-CAATGCCAATTTCTGAGGCATTAC) (SEQ ID NO:24), was complementary to the DNA binding domain. The expected size of PCR fragments was 1.5 kb.

PCR amplifications

[0179] All reactions were performed in 50 μ l in an automated Eppendorf Mastercycler Gradient thermocycler (PerkinElmer, Boston, MA). Each reaction mixture contained 200 ng of template DNA, 1 μ M each oligonucleotide primer, 1 mM Mn(Oac)₂, 200 μ M each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), and 1.0 unit of rTth polymerase, XL (Applied Biosystems, Foster City, CA) in 1 x XL Buffer II. Ampliwax PCR gem 100 was used to facilitate hot start (Applied Biosystems, Foster City, CA).

[0180] Cycling conditions were as follows: 2 min of denaturation at 94° C, followed by 35 cycles of 15 s of denaturation at 94° C, 30 s of annealing at 45° C, and 2 min of elongation at 72° C.

[0181] PCR products (10 μ l) were electrophoretically separated in a 1% NuSieve agarose gel (FMC BioProducts, Rockland, MN), stained with ethidium bromide, and visualized by UV light. DNA molecular markers were used on each gel to facilitate the determination of the sizes of the reaction products.

[0182] To control for specificity of the assay, PCR was also performed with 100 ng of DNA from a plasmid containing AAV2 sequences.

DNA sequencing

[0183] PCR products were purified on 1% low-melting agarose gels (FMC Bioproducts, Rockland, ME), and the sequences were determined using primers designed from AAV-5 sequences.

[0184] Sequence data was analyzed with the NTI vector suite software package (InforMax, Frederick, MD).

[0185] Virus preparations from different cell cultures and passages were processed individually for DNA extraction and PCR analysis. PCR amplification using primers forward 1 and reverse 2 revealed the presence of parvovirus-like sequences in all four preparations. Sequence analysis revealed the presence of AAV sequences. The VP1 ORF of caprine AAV, corresponding to nucleotides 2,207 to 4,381 of AAV-5 genome, has 93% nucleotide identity (2,104/2,266, Gaps 6/2,266) with primate AAV-5 (see Figures 12A-12B) isolated from humans (J. Virol 1999; 73:1309-1319). Protein comparison showed 94% identity (682/726) and 96% similarity (698/726) between the primate AAV-5 and caprine AAV VP1 proteins (see, Figure 13). Most if not all mutations appeared to be on the surface (see, Figure 15). Figure 16 shows the predicted location of the surface amino acids that differ between AAV-5 and caprine AAV, based on the surface structure of the AAV-2 capsid. The 3 filled triangles represent insertions in caprine AAV, relative to AAV-2, that are likely to be located on the surface.

[0186] Without being bound by a particular theory, surface mutations were probably driven by selective pressure due to the humoral immune system and/or adaptation to ruminant receptors. The lack of changes in non-surface exposed areas may imply a lack of pressure from the cellular immune response. These mutated regions in the caprine virus may improve the resistance to pre-existing human anti-AAVS antibodies.

[0187] The caprine AAV sequence was compared to other AAV serotypes and these serotypes were compared with each other in order to analyze the differences in the non-conserved region. In particular, Figures 14A-14H show a comparison of the amino acid sequence of VP1 from primate AAV-1, AAV-2, AAV-3B, AAV-4, AAV-6, AAV-8, AAV-5 and caprine AAV. Conserved amino acids in the sequences are indicated by * and the accessibility of the various amino acid positions based on the crystal structure is shown. B indicates that the amino acid is buried between the inside and outside surface. I indicates the amino acid is found on the inside surface and O indicates the amino acid is found on the outside surface.

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[0188] The non-conserved region between AAV-5 and caprine AAV includes 43 mutations. 17 of these 43 mutations are non-conserved between AAV-2 and AAV-8. Only one of these mutations originated in the same amino acid in caprine AAV and AAV-8. The non-conserved region between AAV-5 and caprine AAV includes 348 amino acids. This non-conserved region is compressed to 157 amino acids when analyzing the region containing the 17 joint mutations.

[0189] Tables 8-11 show the results of the comparisons.

Table 8

Mutations in surface (O) residues of AAV-2 vs. AAV-8 and AAV-5 vs. Caprine - AAV		
Region	AAV-2 vs. AAV-8 mutations (x)/surface residues (O)	AAV-5 vs. Caprine-AAV mutations (*) /surface residues (O)
100-200	04/19 (+2 insertions)	00/19
200-300	01/20	01/20
300-400	16/31	03/30
400-500	20/46 (+1 insertion)	11/43 (+1 insertion)
500-600	13/27	04/30
700-750	05/24	01/24
100-750	59/167 (35%) 65% identity	20/167 (12%) 88% identity

Table 9

Mutations in surface (O) residues of AAV-2 vs. all AAVs.									
Region	AAV2 vs. AAV1 mut/surface	AAV2 vs. AAV3a mut/surface	AAV2 vs. AAV4 mut/surface	AAV2 vs. AAV5 mut/surface	AAV2 vs. AAV6 mut/surface	AAV2 vs. AAV7 mut/surface	AAV2 vs. AAV8 mut/surface	AAV2 vs. Caprine AAV mut/surface	
100-200	01/19 (1 ins)	00/19	08/19 (3 del)	10/19 (1 ins)	01/19	05/19 (1 ins)	04/19 (2 ins)	10/19 (1 ins)	
200-300	02/20	02/20	07/20 (3 ins)	06/20 (2 ins)	01/20	03/20 (1 ins)	01/20	06/20 (2 ins)	
300-400	15/31	11/31	24/31	17/30 (6 del)	17/31	14/31	16/31	18/30 (6 del)	
400-500	21/46	14/46	36/46 (ins, del)	36/44 (3 ins)	21/46	22/46 (1 del)	20/46 (1 ins)	37/44 (3 ins)	
500-600	10/27	07/27	15/27	15/30	10/27	10/27	13/27	17/30	
700-750	06/24	00/24	13/24	11/24	06/24	07/24	05/24	11/24	
100-750	55/167 (33%) 67% identity	34/167 (20%) 80% identity	103/167 (62%) 38% identity	95/167 (57%) 43% identity	56/167 (34%) 66% identity	61/167 (37%) 63% identity	59/167 (35%) 65% identity	99/167 (59%) 41% identity	

Table 10

Surface identity (%)	AAV1	AAV3a	AAV4	AAV5	AAV6	AAV7	AAV8	Caprine AAV
AAV2	67	80	38	43	66	63	65	41
AAV5								88

Table 11

Capsid similarity (%)	AAV1	AAV3a	AAV4	AAV5	AAV6	AAV7	AAV8	Caprine AAV
AAV2	83	87	59	56	83	82	83	56

EXAMPLE 6IMMUNOREACTIVITY OF CAPRINE AAV AND COMPARISON TO OTHER AAVSA. Neutralization Activity of Primate AAV Serotypes

[0190] The neutralization activity of the primate AAV serotypes indicated in Table 12 was assessed using the methods described above. Immunoreactivity was determined using a purified pooled human IgG (designated IVIg 8 in Tables 12 and 13).

[0191] As shown in Tables 12 and 13, most serotypes were neutralized by the pooled human IgG. at clinically relevant concentrations. AAV-4 and AAV-8 were more resistant to neutralization than AAV-1, AAV-2 and AAV-6, which were more resistant to neutralization than AAV-3, which was more resistant to neutralization than AAV-5.

B. Neutralization Activity of Caprine AAV vs. Primate AAV Serotypes

[0192] The neutralization activity of goat AAV was compared to primate AAV-5 using the methods described above. Immunoreactivity was determined using a purified pooled human IgG (designated IVIg 8 in Table 14). As shown in Table 14, caprine AAV displayed more resistance to neutralization than AAV-5. Table 14 also shows the neutralization activity of AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6 and AAV-8, as determined in the above example, relative to the caprine AAV.

[0193] In another experiment, the neutralization activity of caprine AAV relative to AAV-8 was examined using three different purified pools of human IgG, designated IVIg 3, IVIg 6 and IVIg 8, respectively, in Tables 15 and 16. As shown in the tables, caprine AAV was more resistant to neutralization than AAV-8 using all three pools of human IgG.

TABLE 12

IVIg 8 (ug/ul)	50	10	1	0.1	0.01
Vector					
AAV 1	0	14		86	100
AAV 2	0	2		100	100
AAV 3	0	0	45		92
AAV 4	49		100	100	100
AAV 5	0	1	1	17	
AAV 6	0	14		81	100
AAV 8	0		77	88	90

The first concentration for each serotype showing >50% blue cells compared to the control is highlighted.

TABLE 13

Vector	Lowest concentration of IVIG (mg/ml) showing >50% neutralization of the virus
	IVIG (Panglobulin, ZLB Bioplasma, lot# 1838-00351))
AAV1	10
AAV2	10
AAV3B	1
AAV4	50
AAV5	0.1
AAV6	10

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(continued)

Vector	Lowest concentration of IVIG (mg/ml) showing >50% neutralization of the virus
	IVIG (Panglobulin, ZLB Bioplasma, lot# 1838-00351))
AAV8	50

TABLE 14

Vector	Lowest concentration of IVIG (mg/ml) showing >50% neutralization of the virus
	IVIG (Panglobulin, ZLB Bioplasma, lot# 1838-00351))
AAV5	0.1
Caprine-AAV	50

TABLE 15

IVIg 3 (ug/ul) Vector	50	40	20	10	1	0.1
AAV 8	1	2	32	33		105
Goat AAV	16	32		69	92	102

IVIg 6 (ug/ul) Vector	50	40	20	10	1	0.1
AAV 8	0	0	32	22		110
Goat AAV	10	14		113	125	126

IVIg 8 (ug/ul) Vector	50	40	20	10	1	0.1
AAV 8	0	0	11	44		105
Goat AAV	8	14		80	93	120

TABLE 16

	Lowest concentration of IVIG (mg/ml) showing >50% neutralization of the virus.		
	IVIG (Panglobulin, ZLB Bioplasma, lot# 1838-00299)	IVIG (Panglobulin, ZLB Bioplasma, lot# 1838-00351)	IVIG (Baxter, Polygam S/D, lot# 02J06AX11)
Vector			
AAV8	10	10	10

(continued)

5	Vector	Lowest concentration of IVIG (mg/ml) showing >50% neutralization of the virus.	
		IVIG (Panglobulin, ZLB Bioplasma, lot# 1838-00299)	IVIG (Panglobulin, ZLB Bioplasma, lot# 1838-00351)
	Caprine-AAV	40	40

EXAMPLE 7**AGILITY OF CAPRINE AAV TO TRANSDUCE STRIATAL NEURONS AND GLIAL CELLS AND COMPARISON TO OTHER AAVS**

[0194] In order to examine the ability of the various AAVs to transduce striatal neurons and glial cells, the following experiment was done. Primary cultures of dissociated striatal neurons were prepared from embryonic day 18 Sprague-Dawley rat embryos. Dissected striatal tissue was minced into small pieces and was incubated in trypsin for 30 min. The tissue was then triturated through a Pasteur pipette and cells were plated at a density of 350,000 per well in 12-well culture dishes containing round glass 18 mm coverslips coated with poly-D-lysine. The culture medium was neurobasal medium supplemented with 2% B-27, 0.5 mM L-glutamine and 25 mM L-glutamic acid. Cultures were maintained at 37°C in 5% CO₂ and were used in experiments two to three weeks after dissociation. At this stage, dopaminergic and striatal neurons are distinguished both morphologically and by expression of biological markers.

[0195] The striatal cultures were incubated for five days with 10⁴ MOI rAAV virions derived from AAV-2, AAV-4, AAV-5, AAV-6, AAV-8, and caprine AAV that contained the β -galactosidase gene (LacZ), prepared using the triple transfection method described in Example 1. For caprine AAV, the capsid coding sequence present in pHLP19 (described in U.S. Patent No. 6,001,650) was substituted with the caprine VP1 coding sequence as follows. Briefly, plasmid pHLP19 was digested with *Swa*I and *Age*I (New England Biolabs, Beverly, MA 01915-5599), the fragment of interest was purified on a 1% low-melting agarose gel (FMC Bioproducts, Rockland, ME), and used for ligation with the PCR fragment containing the caprine capsid. The caprine capsid PCR fragment was amplified using a forward primer: AAATCAGGTATGTCTTTT-GTTGATCACCC (SEQ ID NO:27) and a reverse primer: ACACGAATTAACCGGTTTATTGAGGGTATGCGACATGAATGGG (SEQ ID NO:28). The PCR fragment was digested with the enzyme *Age*I (New England Biolabs, Beverly, MA 01915-5599) and used for ligation with the digested plasmid.

[0196] Efficient and sustained expression of the β -gal protein was seen in striatal neurons following transduction with the vectors. Expression efficiency was highest in AAV6 followed by AAV8, AAV2, AAV5, caprine AAV and AAV4. AAV6 transduced neurons exclusively, whereas AAV5-mediated gene transfer was inefficient in neurons but transduced the glial cells. All other vectors transduced both neurons and glial cells.

EXAMPLE 8**ABILITY OF CAPRINE AAV TO TRANSDUCE MUSCLE AND COMPARISON TO OTHER AAVS**

[0197] In order to determine the ability of the various AAVs to transduce muscle in the presence or absence of IVIG, the following experiment was done. Male SCID mice (15-25g) were injected intramuscularly with 2e11 vector genomes of caprine rAAV virions, rAAV-1 virions, or rAAV-8 virions (5 mice per group), each of said virions encoding human factor IX. These virions were made using the triple transfection method described in Example 1. The capsid coding sequence present in pHLP19 was substituted with the caprine VP1 coding sequence as described above. Retro-orbital blood was collected 1, and 2 weeks after vector injection and plasma was extracted. Mice tested with IVIG (Carimune: purified immunoglobulin from a pool of human serum, ZLB Bioplasma, lot# 03287-00117) were injected via the tail vein (250 μ l), 24 hours before the vector injection. Human FIX was measured in the plasma samples using a hFIX ELISA.

[0198] As shown in Figure 17, caprine rAAV virions did not transduce muscle. the rAAV-8 and rAAV-1 virions displayed similar levels of expression of hFIX. AAV-1 was more resistant to neutralization than AAV-8 *in vivo*.

EXAMPLE 9ABILITY OF CAPRICE AAV TO TRANSDUCE LIVER AND COMPARISON TO OTHER AAVS ANY BIODISTRIBUTION OF PROTEINS EXPRESSED FROM GENES DELIVERED BY CAPRINE AAV VIRIONS

[0199] In order to determine the ability of the various AAVs to transduce liver in the presence or absence of IVIG, the following experiment was done. Male SCID mice (15-25g) were injected via the tail vein with 5e11 vector genomes of caprine rAAV virions or rAAV-8 virions (5 mice per group). The virions included the gene encoding human factor IX (hFIX). The rAAV-2 virion data below was from another experiment. In particular, the virions were generated using plasmid pAAV-hFIX16, containing the human factor IX gene under the control of a liver-specific promoter (described in Miao et al., Mol. Ther. (2000) 1:522-532). Plasmid pAAV-hFIX16 is an 11,277 bp plasmid encoding a human Factor IX minigene. In this construct, the FIX cDNA is interrupted between exons 1 and 2 with a deleted form of intron 1 which has been shown to increase expression of FIX. FIX expression is under the transcriptional control of the ApoE hepatic control region (HCR) and the human alpha 1 antitrypsin promoter (hAAT), as well as a bovine growth hormone polyadenylation signal (gHPA). The backbone of plasmid pAAV-hFIX16 contains the β -lactamase gene, conferring ampicillin resistance, a bacterial origin of replication, a M13/F1 origin of replication, and a fragment of bacteriophage lambda DNA. The lambda DNA increases the size of the plasmid backbone to 6,966 bp, which prevents its packaging during AAV vector production.

[0200] The recombinant AAV virions were produced using the triple transfection method described above. For the caprine rAAV virions, the VP1 coding sequence present in plasmid pHLP19 was substituted with the caprine VP1 coding sequence as described above.

[0201] After injection, retro-orbital blood was collected 1, 2, 4 (5 mice per group) and 8 weeks (2 mice per group) after injection and plasma was extracted. Mice tested with IVIG (Panglobulin: purified immunoglobulin from a pool of human serum, ZLB Bioplasma, lot# 1838-00299) were injected via the tail vein (250 μ l), 24 hours before the vector injection. Human FIX was measured in the plasma samples by a hFIX ELISA.

[0202] As shown in Figure 18, transduction of liver with the recombinant caprine AAV virions after intravenous administration was low. Higher hFIX expression was seen using the rAAV-8 virions than with the rAAV-2 virions, and rAAV-2 virions showed higher expression than the caprine rAAV virions. The caprine rAAV virions were more resistant to neutralization than the rAAV-2 virions *in vivo*. Human FIX expression was reduced in the caprine rAAV-injected mice with preexisting IVIG neutralizing titers of 120 while the expression of hFIX was completely blocked in the rAAV-2-injected mice with preexisting IVIG neutralizing titers of 10.

[0203] For biodistribution analysis, mice (2 mice per group) were sacrificed and organs were collected 4 weeks after vector injection. Organs collected included brain, testis, muscle (quadriceps), kidney, spleen, lung, heart, and liver. To measure hFIX, quantitative-PCR was done on DNA samples extracted from different tissues. As shown in Figure 19, biodistribution of intravenously-administered caprine rAAV virions in male SCID mice showed that the caprine rAAV virions had lung tropism.

EXAMPLE 10ISOLATION AND CHARACTERIZATION OF A NEW BOVINE AAV

[0204] Evidence of parvovirus contamination was seen in bovine adenovirus (BAV) type 8, strain Misk/67 (available from the ATCC, Manassas, VA, Accession no. VR-769) isolated from calf lungs, using techniques known in the art. This new isolate was named "AAV-C1." AAV-C1 was partially amplified by PCR, and sequenced. Figures 20A and 20B show the nucleotide sequence and amino acid sequence respectively, of VP1 from AAV-C1. The VP1 amino acid sequence from AAV-C1 was compared with other AAV VP1s. In particular, Figures 21A-21H show a comparison of the amino acid sequence of VP1 from AAV-C1 with primate AAV-1, AAV-2, AAV-3B, AAV-4, AAV-6, AAV-8, AAV-5 and caprine AAV. Conserved amino acids in the sequences are indicated by * and the accessibility of the various amino acid positions based on the crystal structure is shown. B indicates that the amino acid is buried between the inside and outside surface. I indicates the amino acid is found on the inside surface and O indicates the amino acid is found on the outside surface.

[0205] VP1 from AAV-C1 displayed approximately 76% identity with AAV-4. AAV-C1 displayed approximately 54% identity with AAV-5 VP1, with high homology in the Rep protein, the first 137 amino acids of AAV-5 VP1 and the non translated region after the stop of AAV-5 VP1 (not shown). Thus, AAV-C1 appears to be a natural hybrid between AAV-5 and AAV-4. AAV-C1 also displayed approximately 58% sequence identity with VP1s from AAV-2 and AAV-8, approximately 59% sequence identity with VP1s from AAV-1 and AAV-6, and approximately 60% sequence identity with VP1 from AAV-3B.

[0206] The sequence differences between AAV-4 and AAV-C1 were scattered throughout the capsid, unlike the differences between AAV-5 and caprine AAV (AAV-G1) wherein the changes were exclusively in the C-terminal hypervar-

able region of VP1. The similarity with the AAV-4 sequence was from the VP2 start to the capsid stop. AAV-C1 appears to be one of the most divergent of the mammalian AAVs with approximately 58% sequence homology with AAV-2. In particular, the bovine AAV described in Schmidt et al. was partially amplified from bovine adenovirus type 2. Comparison of the nucleotide sequence of VP1 from AAV-C1 and the bovine AAV described in Schmidt et al. showed 12 nucleotide changes 5 amino acid differences. These differences occurred at positions 334 (Q substituted for H present in AAV-C1 VP1), 464 (K substituted for N present in AAV-C1 VP1), 465 (T substituted for K present in AAV-C1 VP1), 499 (R substituted for G present in AAV-C1 VP1) and 514 (G substituted for R present in AAV-C1 VP1).

[0207] The full capsid of AAV-C1 was cloned in a plasmid that was used to produce pseudotyped AAV-2 vectors. An AAV-C1 vector containing the LacZ gene (AAV-C1-LacZ) was produced for further characterization, using the triple transfection techniques described above with the exception that the capsid sequence present in pHLP19 was replaced with the bovine capsid sequence. The titer of AAV-C1-LacZ (vg/ml) was calculated using quantitative PCR (Q-PCR) as described above. As shown in Table 17, AAV-C1 LacZ vector was produced efficiently; high titers of vector (2.45e10vg/ml) were detected by Q-PCR. AAV-C1 LacZ vector showed efficient transduction of cells *in vitro* (cells expressing LacZ were present in numbers comparable to other AAVs).

Table 17: Q-PCR analysis of AAV-C1-LacZ vector.

Sample	Average (vg/mL)	Std dev (vg/mL)	% CV
AAV2-lacZ	1.11E+11	1.09E+10	9.9
AAV-C1-lacZ	2.45E+10	1.88E+09	7.7
LacZ reference	9.96E+12	7.11E+11	7.1

EXAMPLE 11

IMMUNOREACTIVITY OF BOVINE AAV AND COMPARISON TO OTHER AAVS

[0208] The neutralization activity of bovine AAV-C1 relative to primate AAV-2 was assessed using the methods described above in Example 6. Immunoreactivity was determined using a purified pooled human IgG (IVIG-8, Panglobulin Lot # 1838-00351, ZLB Bioplasma AG, Berne, Switzerland). Neutralizing assays *in vitro* showed that AAV-C1 was 16 times more resistant to neutralization by human IVIG than AAV-2. The lowest concentration of IVIG (mg/ml) showing more than 50% neutralization of AAV-2 was 0.2 mg/ml while AAV-C1 was 3.25 mg/ml.

[0209] Thus, methods for making and using mutant AAV virions with decreased immunoreactivity are described.

[0210] Preferred instances of the present disclosure are described below and are referred to as instances E1 to E36.

[0211] E1. A mutated adeno-associated virus (AAV) capsid protein that when present in an AAV virion imparts decreased immunoreactivity to the virion as compared to the corresponding wild-type virion.

[0212] E2. The protein of E1, wherein the mutation comprises at least one amino acid substitution, deletion or insertion to the native protein.

[0213] E3. The protein of E2, wherein the mutation comprises at least one amino acid substitution.

[0214] E4. The protein of E3, wherein the at least one amino acid substitution is in the spike or plateau region of the AAV virion surface.

[0215] E5. The protein of E4, wherein the amino acid substitution comprises a substitution of one or more of the amino acids occurring at a position corresponding to a position of the AAV-2 VP2 capsid selected from the group consisting of amino acid 126, 127, 128, 130, 132, 134, 247, 248, 315, 334, 354, 357, 360, 361, 365, 372, 375, 377, 390, 393, 394, 395, 396, 407, 411, 413, 418, 437, 449, 450, 568, 569, and 571.

[0216] E6. The protein of E5, wherein the naturally occurring amino acid at the position is substituted with an alanine.

[0217] E7. The protein of E6, wherein the protein further comprises a substitution of histidine for the amino acid occurring at the position corresponding to the amino acid found at position 360 of AAV-2 VP2.

[0218] E8. The protein of any of E5-7, wherein the protein comprises a substitution of lysine for the amino acid occurring at the position corresponding to the amino acid found at position 571 of AAV-2 VP2.

[0219] E9. A polynucleotide encoding the mutated protein of any of E1-8.

[0220] E10. A recombinant AAV virion comprising the mutated protein of any of claims 1-8.

[0221] E11. The recombinant AAV virion of E10, wherein said virion comprises a heterologous nucleic acid molecule encoding an antisense RNA or a ribozymes.

[0222] E12. The recombinant AAV virion of E10, wherein said virion comprises a heterologous nucleic acid molecule encoding a therapeutic protein operably linked to control elements capable of directing the *in vivo* transcription and translation of said protein.

[0223] E13. Use of a recombinant AAV virion of either of E11 or 12, to deliver a heterologous nucleic acid molecule to a cell or tissue of a vertebrate subject, whereby the protein encoded by the heterologous nucleic acid molecule is expressed at a level that provides a therapeutic effect.

[0224] E14. The use of E13, wherein said cell or tissue is a muscle cell or tissue.

[0225] E15. The use of E14, wherein said muscle cell or tissue is derived from skeletal muscle.

[0226] E16. The use of E13, wherein said recombinant AAV virion is delivered into said cell or tissue *in vivo*.

[0227] E17. The use of E16, wherein said recombinant AAV virion is delivered by intramuscular injection.

[0228] E18. The use of E13, wherein said recombinant AAV virion is delivered into said cell or tissue *in vitro*.

[0229] E19. The use of E13, wherein said recombinant AAV virion is delivered into the bloodstream.

[0230] E20. The use of E19, wherein said recombinant AAV virion is delivered intravenously.

[0231] E21. The use of E19, wherein said recombinant AAV virion is delivered intraarterially.

[0232] E22. The use of E13, wherein said recombinant AAV virion is delivered to the liver.

[0233] E23. The use of E13, wherein said recombinant AAV virion is delivered to the brain.

[0234] E24. A method of delivering a recombinant AAV virion to a cell or tissue of a vertebrate subject, said method comprising:

(a) providing a recombinant AAV virion according to E12;

(b) delivering said recombinant AAV virion to said cell or tissue, whereby said protein is expressed at a level that provides a therapeutic effect.

[0235] E25. Use of a recombinant adeno-associated virus (AAV) virion to deliver a heterologous nucleic acid encoding a protein to a cell or tissue of a vertebrate subject, whereby the protein is expressed at a level that provides a therapeutic effect, wherein said recombinant AAV virion comprises

(i) a non-primate, mammalian AAV capsid protein that when present in an AAV virion imparts decreased immunoreactivity to the virion as compared to immunoreactivity of primate AAV-2; and

(ii) said heterologous nucleic acid molecule, wherein said heterologous nucleic acid molecule encodes a therapeutic protein and is operably linked to control elements capable of directing the *in vivo* transcription and translation of said protein.

[0236] E26. The use of E25, wherein said cell or tissue is a muscle cell or tissue.

[0237] E27. The method of E26, wherein said muscle cell or tissue is derived from skeletal muscle.

[0238] E28. The use of E25, wherein said recombinant AAV virion is delivered into said cell or tissue *in vivo*.

[0239] E29. The method of E26, wherein said recombinant AAV virion is delivered by intramuscular injection.

[0240] E30. The use of E25, wherein said recombinant AAV virion is delivered into said cell or tissue *in vitro*.

[0241] E31. The use of E25, wherein said recombinant AAV virion is delivered into the bloodstream.

[0242] E32. The use of E31, wherein said recombinant AAV virion is delivered intravenously.

[0243] E33. The method of E31, wherein said recombinant AAV virion is delivered intraarterially.

[0244] E34. The use of E25, wherein said recombinant AAV virion is delivered to the liver.

[0245] E35. The use of E25, wherein said recombinant AAV virion is delivered to the brain.

[0246] E36. A method of delivering a recombinant AAV virion to a cell or tissue of a vertebrate subject, said method comprising:

(a) providing a recombinant AAV virion, wherein said AAV virion comprises

(i) a non-primate, mammalian adeno-associated virus (AAV) capsid protein that when present in an AAV virion imparts decreased immunoreactivity to the virion as compared to immunoreactivity of primate AAV-2; and

(ii) a heterologous nucleic acid molecule encoding a therapeutic protein operably linked to control elements capable of directing the *in vivo* transcription and translation of said protein;

(b) delivering said recombinant AAV virion to said cell or tissue, whereby said protein is expressed at a level that provides a therapeutic effect.

SEQUENCE LISTING

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40	Pro	Ser	Thr	Thr	Phe	Ser	Ala	Ala	Lys	Phe	Ala	Ser	Phe	Ile	Thr	Gln
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50	Glu	Asn	Ser	Lys	Arg	Trp	Asn	Pro	Glu	Ile	Gln	Tyr	Thr	Ser	Asn	Tyr
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	cagtacagca ccgggcaggt caccgtggag atggagtggg agctcaagaa ggaaaactcc	2040
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10	Asn	Arg	Ala	Asp	Glu	Val	Ala	Arg	Glu	His	Asp	Ile	Ser	Tyr	Asn	Glu
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		210					215					220				
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65	Ser	Tyr	Asn	Asn	His	Gln	Tyr	Arg	Glu	Ile	Lys	Ser	Gly	Ser	Val	Asp
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40	Phe	Ala	Pro	Ser	Gln	Asn	Leu	Phe	Lys	Leu	Ala	Asn	Pro	Leu	Val	Asp
				420					425					430		
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	Phe	Asn	Lys	Asn	Leu	Ala	Gly	Arg	Tyr	Ala	Asn	Thr	Tyr	Lys	Asn	Trp
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	465					470					475					480
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[illegible]

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Pro Asn Gln Gln His Gln Asp Gln Ala Arg Gly Leu Val Leu Pro Gly
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Tyr Asn Tyr Leu Gly Pro Gly Asn Gly Leu Asp Arg Gly Glu Pro Val
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Asn Arg Ala Asp Glu Val Ala Arg Glu His Asp Ile Ser Tyr Asn Glu
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Gln Leu Glu Ala Gly Asp Asn Pro Tyr Leu Lys Tyr Asn His Ala Asp
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Ala Glu Phe Gln Glu Lys Leu Ala Asp Asp Thr Ser Phe Gly Gly Asn
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Leu Gly Lys Ala Val Phe Gln Ala Lys Lys Arg Val Leu Glu Pro Phe
 115 120 125

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Gly Leu Val Glu Glu Gly Ala Lys Thr Ala Pro Thr Gly Lys Arg Ile
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Asp Asp His Phe Pro Lys Arg Lys Lys Ala Arg Thr Glu Glu Asp Ser
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Lys Pro Ser Thr Ser Ser Asp Ala Glu Ala Gly Pro Ser Gly Ser Gln
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			195					200					205			
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			435					440					445								
15	Phe	Gln	Lys	Asn	Leu	Ala	Gly	Arg	Tyr	Ala	Asn	Thr	Tyr	Lys	Asn	Trp					
		450					455					460									
20	Phe	Pro	Gly	Pro	Met	Gly	Arg	Thr	Gln	Gly	Trp	Asn	Thr	Ser	Ser	Gly					
	465					470					475					480					
25	Ser	Ser	Thr	Asn	Arg	Val	Ser	Val	Asn	Asn	Phe	Ser	Val	Ser	Asn	Arg					
				485						490					495						
30	Met	Asn	Leu	Glu	Gly	Ala	Ser	Tyr	Gln	Val	Asn	Pro	Gln	Pro	Asn	Gly					
			500						505					510							
35	Met	Thr	Asn	Thr	Leu	Gln	Gly	Ser	Asn	Arg	Tyr	Ala	Leu	Glu	Asn	Thr					
			515					520					525								
40	Met	Ile	Phe	Asn	Ala	Gln	Asn	Ala	Thr	Pro	Gly	Thr	Thr	Ser	Val	Tyr					
		530					535					540									
45	Pro	Glu	Asp	Asn	Leu	Leu	Leu	Thr	Ser	Glu	Ser	Glu	Thr	Gln	Pro	Val					
	545					550					555					560					
50	Asn	Arg	Val	Ala	Tyr	Asn	Thr	Gly	Gly	Gln	Met	Ala	Thr	Asn	Ala	Gln					
				565						570					575						
55	Asn	Ala	Thr	Thr	Ala	Pro	Thr	Val	Gly	Thr	Tyr	Asn	Leu	Gln	Glu	Val					
				580					585					590							
60	Leu	Pro	Gly	Ser	Val	Trp	Met	Glu	Arg	Asp	Val	Tyr	Leu	Gln	Gly	Pro					
			595					600					605								
65	Ile	Trp	Ala	Lys	Ile	Pro	Glu	Thr	Gly	Ala	His	Phe	His	Pro	Ser	Pro					
		610					615					620									
70	Ala	Met	Gly	Gly	Phe	Gly	Leu	Lys	His	Pro	Pro	Pro	Met	Met	Leu	Ile					
	625					630					635					640					

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	Lys Asn Thr Pro Val Pro Gly Asn Ile Thr Ser Phe Ser Asp Val Pro	
	645	650 655
5	Val Ser Ser Phe Ile Thr Gln Tyr Ser Thr Gly Gln Val Thr Val Glu	
	660	665 670
10	Met Glu Trp Glu Leu Lys Lys Glu Asn Ser Lys Arg Trp Asn Pro Glu	
	675	680 685
15	Ile Gln Tyr Thr Asn Asn Tyr Asn Asp Pro Gln Phe Val Asp Phe Ala	
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	20	25 30
40	Lys Ala Ala Glu Arg His Lys Asp Asp Ser Arg Gly Leu Val Leu Pro	
	35	40 45
	Gly Tyr Lys Tyr Leu Gly Pro Gly Asn Gly Leu Asp Lys Gly Glu Pro	
	50	55 60
45	Val Asn Glu Ala Asp Ala Ala Ala Leu Glu His Asp Lys Ala Tyr Asp	
	65	70 75 80
50	Gln Gln Leu Lys Ala Gly Asp Asn Pro Tyr Leu Lys Tyr Asn His Ala	
	85	90 95
55		

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	Asp	Ala	Glu	Phe	Gln	Glu	Arg	Leu	Gln	Glu	Asp	Thr	Ser	Phe	Gly	Gly
				100					105					110		
5	Asn	Leu	Gly	Arg	Ala	Val	Phe	Gln	Ala	Lys	Lys	Arg	Ile	Leu	Glu	Pro
			115					120					125			
10	Leu	Gly	Leu	Val	Glu	Glu	Ala	Ala	Lys	Thr	Ala	Pro	Gly	Lys	Lys	Arg
		130					135					140				
15	Pro	Val	Asp	Gln	Ser	Pro	Gln	Glu	Pro	Asp	Ser	Ser	Ser	Gly	Val	Gly
	145					150					155					160
20	Lys	Ser	Gly	Lys	Gln	Pro	Ala	Arg	Lys	Arg	Leu	Asn	Phe	Gly	Gln	Thr
					165					170					175	
25	Gly	Asp	Ser	Glu	Ser	Val	Pro	Asp	Pro	Gln	Pro	Leu	Gly	Glu	Pro	Pro
				180					185					190		
30	Ala	Ala	Pro	Thr	Ser	Leu	Gly	Ser	Asn	Thr	Met	Ala	Ser	Gly	Gly	Gly
			195					200					205			
35	Ala	Pro	Met	Ala	Asp	Asn	Asn	Glu	Gly	Ala	Asp	Gly	Val	Gly	Asn	Ser
		210					215					220				
40	Ser	Gly	Asn	Trp	His	Cys	Asp	Ser	Gln	Trp	Leu	Gly	Asp	Arg	Val	Ile
	225					230					235					240
45	Thr	Thr	Ser	Thr	Arg	Thr	Trp	Ala	Leu	Pro	Thr	Tyr	Asn	Asn	His	Leu
					245					250					255	
50	Tyr	Lys	Gln	Ile	Ser	Ser	Gln	Ser	Gly	Ala	Ser	Asn	Asp	Asn	His	Tyr
				260					265					270		
55	Phe	Gly	Tyr	Ser	Thr	Pro	Trp	Gly	Tyr	Phe	Asp	Phe	Asn	Arg	Phe	His
			275					280					285			
60	Cys	His	Phe	Ser	Pro	Arg	Asp	Trp	Gln	Arg	Leu	Ile	Asn	Asn	Asn	Trp
	290						295					300				
65	Gly	Phe	Arg	Pro	Lys	Lys	Leu	Ser	Phe	Lys	Leu	Phe	Asn	Ile	Gln	Val
	305					310					315					320
70	Lys	Glu	Val	Thr	Gln	Asn	Asp	Gly	Thr	Thr	Thr	Ile	Ala	Asn	Asn	Leu

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				325					330					335		
5	Thr	Ser	Thr	Val	Gln	Val	Phe	Thr	Asp	Ser	Glu	Tyr	Gln	Leu	Pro	Tyr
				340					345					350		
10	Val	Leu	Gly	Ser	Ala	His	Gln	Gly	Cys	Leu	Pro	Pro	Phe	Pro	Ala	Asp
			355					360					365			
15	Val	Phe	Met	Val	Pro	Gln	Tyr	Gly	Tyr	Leu	Thr	Leu	Asn	Asn	Gly	Ser
		370					375					380				
20	Gln	Ala	Val	Gly	Arg	Ser	Ser	Phe	Tyr	Cys	Leu	Glu	Tyr	Phe	Pro	Ser
						390					395					400
25	Gln	Met	Leu	Arg	Thr	Gly	Asn	Asn	Phe	Gln	Phe	Ser	Tyr	Thr	Phe	Glu
					405					410					415	
30	Asp	Val	Pro	Phe	His	Ser	Ser	Tyr	Ala	His	Ser	Gln	Ser	Leu	Asp	Arg
				420					425					430		
35	Leu	Met	Asn	Pro	Leu	Ile	Asp	Gln	Tyr	Leu	Tyr	Tyr	Leu	Asn	Arg	Thr
			435					440					445			
40	Gln	Gly	Thr	Thr	Ser	Gly	Thr	Thr	Asn	Gln	Ser	Arg	Leu	Leu	Phe	Ser
		450					455					460				
45	Gln	Ala	Gly	Pro	Gln	Ser	Met	Ser	Leu	Gln	Ala	Arg	Asn	Trp	Leu	Pro
						470					475					480
50	Gly	Pro	Cys	Tyr	Arg	Gln	Gln	Arg	Val	Ser	Lys	Thr	Ala	Asn	Asp	Asn
					485					490					495	
55	Asn	Asn	Ser	Asn	Phe	Pro	Trp	Thr	Ala	Ala	Ser	Lys	Tyr	His	Leu	Asn
				500					505					510		
60	Gly	Arg	Asp	Ser	Leu	Val	Asn	Pro	Gly	Pro	Ala	Met	Ala	Ser	His	Lys
			515					520					525			
65	Asp	Asp	Glu	Glu	Lys	Phe	Phe	Pro	Met	His	Gly	Asn	Leu	Ile	Phe	Gly
		530					535					540				
70	Lys	Glu	Gly	Thr	Thr	Ala	Ser	Asn	Ala	Glu	Leu	Asp	Asn	Val	Met	Ile
		545				550					555					560

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	Thr	Asp	Glu	Glu	Glu	Ile	Arg	Thr	Thr	Asn	Pro	Val	Ala	Thr	Glu	Gln
					565					570					575	
5	Tyr	Gly	Thr	Val	Ala	Asn	Asn	Leu	Gln	Ser	Ser	Asn	Thr	Ala	Pro	Thr
				580					585					590		
10	Thr	Arg	Thr	Val	Asn	Asp	Gln	Gly	Ala	Leu	Pro	Gly	Met	Val	Trp	Gln
			595					600					605			
15	Asp	Arg	Asp	Val	Tyr	Leu	Gln	Gly	Pro	Ile	Trp	Ala	Lys	Ile	Pro	His
		610					615					620				
20	Thr	Asp	Gly	His	Phe	His	Pro	Ser	Pro	Leu	Met	Gly	Gly	Phe	Gly	Leu
	625					630					635					640
25	Lys	His	Pro	Pro	Pro	Gln	Ile	Met	Ile	Lys	Asn	Thr	Pro	Val	Pro	Ala
					645					650					655	
30	Asn	Pro	Pro	Thr	Thr	Phe	Ser	Pro	Ala	Lys	Phe	Ala	Ser	Phe	Ile	Thr
				660					665					670		
35	Gln	Tyr	Ser	Thr	Gly	Gln	Val	Ser	Val	Glu	Ile	Glu	Trp	Glu	Leu	Gln
			675					680					685			
40	Lys	Glu	Asn	Ser	Lys	Arg	Trp	Asn	Pro	Glu	Ile	Gln	Tyr	Thr	Ser	Asn
		690					695					700				
45	Tyr	Asn	Lys	Ser	Val	Asn	Val	Asp	Phe	Thr	Val	Asp	Thr	Asn	Gly	Val
	705					710					715					720
50	Tyr	Ser	Glu	Pro	Arg	Pro	Ile	Gly	Thr	Arg	Tyr	Leu	Thr	Arg	Asn	Leu
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	Glu	Gly	Ile	Arg	Glu	Trp	Trp	Asp	Leu	Lys	Pro	Gly	Ala	Pro	Lys	Pro	
			20						25					30			
5	Lys	Ala	Asn	Gln	Gln	Lys	Gln	Asp	Asp	Gly	Arg	Gly	Leu	Val	Leu	Pro	
			35					40					45				
10	Gly	Tyr	Lys	Tyr	Leu	Gly	Pro	Phe	Asn	Gly	Leu	Asp	Lys	Gly	Glu	Pro	
		50					55					60					
15	Val	Asn	Ala	Ala	Asp	Ala	Ala	Ala	Leu	Glu	His	Asp	Lys	Ala	Tyr	Asp	
	65					70					75					80	
	Gln	Gln	Leu	Lys	Ala	Gly	Asp	Asn	Pro	Tyr	Leu	Arg	Tyr	Asn	His	Ala	
					85					90					95		
20	Asp	Ala	Glu	Phe	Gln	Glu	Arg	Leu	Gln	Glu	Asp	Thr	Ser	Phe	Gly	Gly	
				100					105					110			
25	Asn	Leu	Gly	Arg	Ala	Val	Phe	Gln	Ala	Lys	Lys	Arg	Val	Leu	Glu	Pro	
			115					120					125				
30	Phe	Gly	Leu	Val	Glu	Glu	Gly	Ala	Lys	Thr	Ala	Pro	Gly	Lys	Lys	Arg	
		130					135					140					
	Pro	Val	Glu	Gln	Ser	Pro	Gln	Glu	Pro	Asp	Ser	Ser	Ser	Gly	Ile	Gly	
	145					150					155					160	
35	Lys	Thr	Gly	Gln	Gln	Pro	Ala	Lys	Lys	Arg	Leu	Asn	Phe	Gly	Gln	Thr	
					165					170					175		
40	Gly	Asp	Ser	Glu	Ser	Val	Pro	Asp	Pro	Gln	Pro	Leu	Gly	Glu	Pro	Pro	
				180					185					190			
45	Ala	Thr	Pro	Ala	Ala	Val	Gly	Pro	Thr	Thr	Met	Ala	Ser	Gly	Gly	Gly	
			195					200					205				
	Ala	Pro	Met	Ala	Asp	Asn	Asn	Glu	Gly	Ala	Asp	Gly	Val	Gly	Asn	Ala	
		210					215					220					
50	Ser	Gly	Asn	Trp	His	Cys	Asp	Ser	Thr	Trp	Leu	Gly	Asp	Arg	Val	Ile	
	225					230					235					240	
55	Thr	Thr	Ser	Thr	Arg	Thr	Trp	Ala	Leu	Pro	Thr	Tyr	Asn	Asn	His	Leu	

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	245										250										255									
5	Tyr	Lys	Gln	Ile	Ser	Ser	Ala	Ser	Thr	Gly	Ala	Ser	Asn	Asp	Asn	His														
				260						265				270																
10	Tyr	Phe	Gly	Tyr	Ser	Thr	Pro	Trp	Gly	Tyr	Phe	Asp	Phe	Asn	Arg	Phe														
			275					280					285																	
15	His	Cys	His	Phe	Ser	Pro	Arg	Asp	Trp	Gln	Arg	Leu	Ile	Asn	Asn	Asn														
		290					295					300																		
20	Trp	Gly	Phe	Arg	Pro	Lys	Arg	Leu	Asn	Phe	Lys	Leu	Phe	Asn	Ile	Gln														
	305					310					315					320														
25	Val	Lys	Glu	Val	Thr	Thr	Asn	Asp	Gly	Val	Thr	Thr	Ile	Ala	Asn	Asn														
					325					330					335															
30	Leu	Thr	Ser	Thr	Val	Gln	Val	Phe	Ser	Asp	Ser	Glu	Tyr	Gln	Leu	Pro														
				340					345					350																
35	Tyr	Val	Leu	Gly	Ser	Ala	His	Gln	Gly	Cys	Leu	Pro	Pro	Phe	Pro	Ala														
			355					360					365																	
40	Asp	Val	Phe	Met	Ile	Pro	Gln	Tyr	Gly	Tyr	Leu	Thr	Leu	Asn	Asn	Gly														
		370					375					380																		
45	Ser	Gln	Ala	Val	Gly	Arg	Ser	Ser	Phe	Tyr	Cys	Leu	Glu	Tyr	Phe	Pro														
	385					390					395					400														
50	Ser	Gln	Met	Leu	Arg	Thr	Gly	Asn	Asn	Phe	Thr	Phe	Ser	Tyr	Thr	Phe														
					405					410					415															
55	Glu	Asp	Val	Pro	Phe	His	Ser	Ser	Tyr	Ala	His	Ser	Gln	Ser	Leu	Asp														
				420					425				430																	
60	Arg	Leu	Met	Asn	Pro	Leu	Ile	Asp	Gln	Tyr	Leu	Tyr	Tyr	Leu	Asn	Arg														
			435					440					445																	
65	Thr	Gln	Asn	Gln	Ser	Gly	Ser	Ala	Gln	Asn	Lys	Asp	Leu	Leu	Phe	Ser														
		450					455					460																		
70	Arg	Gly	Ser	Pro	Ala	Gly	Met	Ser	Val	Gln	Pro	Lys	Asn	Trp	Leu	Pro														
	465					470					475					480														

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					485					490					495	
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				500					505					510		
10	Gly	Arg	Glu	Ser	Ile	Ile	Asn	Pro	Gly	Thr	Ala	Met	Ala	Ser	His	Lys
			515					520					525			
15	Asp	Asp	Lys	Asp	Lys	Phe	Phe	Pro	Met	Ser	Gly	Val	Met	Ile	Phe	Gly
		530					535					540				
20	Lys	Glu	Ser	Ala	Gly	Ala	Ser	Asn	Thr	Ala	Leu	Asp	Asn	Val	Met	Ile
	545					550					555					560
25	Thr	Asp	Glu	Glu	Glu	Ile	Lys	Ala	Thr	Asn	Pro	Val	Ala	Thr	Glu	Arg
					565					570					575	
30	Phe	Gly	Thr	Val	Ala	Val	Asn	Leu	Gln	Ser	Ser	Ser	Thr	Asp	Pro	Ala
				580					585					590		
35	Thr	Gly	Asp	Val	His	Val	Met	Gly	Ala	Leu	Pro	Gly	Met	Val	Trp	Gln
			595					600					605			
40	Asp	Arg	Asp	Val	Tyr	Leu	Gln	Gly	Pro	Ile	Trp	Ala	Lys	Ile	Pro	His
		610					615					620				
45	Thr	Asp	Gly	His	Phe	His	Pro	Ser	Pro	Leu	Met	Gly	Gly	Phe	Gly	Leu
	625					630					635					640
50	Lys	His	Pro	Pro	Pro	Gln	Ile	Leu	Ile	Lys	Asn	Thr	Pro	Val	Pro	Ala
					645					650					655	
55	Asn	Pro	Pro	Ala	Glu	Phe	Ser	Ala	Thr	Lys	Phe	Ala	Ser	Phe	Ile	Thr
				660					665					670		
60	Gln	Tyr	Ser	Thr	Gly	Gln	Val	Ser	Val	Glu	Ile	Glu	Trp	Glu	Leu	Gln
			675					680					685			
65	Lys	Glu	Asn	Ser	Lys	Arg	Trp	Asn	Pro	Glu	Val	Gln	Tyr	Thr	Ser	Asn
		690					695					700				
70	Tyr	Ala	Lys	Ser	Ala	Asn	Val	Asp	Phe	Thr	Val	Asp	Asn	Asn	Gly	Leu
	705					710					715					720
75	Tyr	Thr	Glu	Pro	Arg	Pro	Ile	Gly	Thr	Arg	Tyr	Leu	Thr	Arg	Pro	Leu
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Glu Gly Ile Arg Glu Trp Trp Asp Leu Lys Pro Gly Ala Pro Lys Pro
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20

Lys Ala Asn Gln Gln Lys Gln Asp Asp Gly Arg Gly Leu Val Leu Pro
 35 40 45

25

Gly Tyr Lys Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys Gly Glu Pro
 50 55 60

Val Asn Ala Ala Asp Ala Ala Ala Leu Glu His Asp Lys Ala Tyr Asp
 65 70 75 80

30

Gln Gln Leu Lys Ala Gly Asp Asn Pro Tyr Leu Arg Tyr Asn His Ala
 85 90 95

Asp Ala Glu Phe Gln Glu Arg Leu Gln Glu Asp Thr Ser Phe Gly Gly
 100 105 110

35

Asn Leu Gly Arg Ala Val Phe Gln Ala Lys Lys Arg Val Leu Glu Pro
 115 120 125

40

Leu Gly Leu Val Glu Glu Gly Ala Lys Thr Ala Pro Gly Lys Lys Arg
 130 135 140

Pro Val Glu Gln Ser Pro Gln Glu Pro Asp Ser Ser Ser Gly Ile Gly
 145 150 155 160

45

Lys Thr Gly Gln Gln Pro Ala Lys Lys Arg Leu Asn Phe Gly Gln Thr

50

55

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				180					185					190			
10	Ala	Thr	Pro	Ala	Ala	Val	Gly	Pro	Thr	Thr	Met	Ala	Ser	Gly	Gly	Gly	
			195					200					205				
15	Ala	Pro	Met	Ala	Asp	Asn	Asn	Glu	Gly	Ala	Asp	Gly	Val	Gly	Asn	Ala	
		210					215					220					
20	Ser	Gly	Asn	Trp	His	Cys	Asp	Ser	Thr	Trp	Leu	Gly	Asp	Arg	Val	Ile	
	225					230					235					240	
25	Thr	Thr	Ser	Thr	Arg	Thr	Trp	Ala	Leu	Pro	Thr	Tyr	Asn	Asn	His	Leu	
					245					250					255		
30	Tyr	Lys	Gln	Ile	Ser	Ser	Ala	Ser	Thr	Gly	Ala	Ser	Asn	Asp	Asn	His	
				260					265					270			
35	Tyr	Phe	Gly	Tyr	Ser	Thr	Pro	Trp	Gly	Tyr	Phe	Asp	Phe	Asn	Arg	Phe	
			275					280					285				
40	His	Cys	His	Phe	Ser	Pro	Arg	Asp	Trp	Gln	Arg	Leu	Ile	Asn	Asn	Asn	
		290					295					300					
45	Trp	Gly	Phe	Arg	Pro	Lys	Arg	Leu	Asn	Phe	Lys	Leu	Phe	Asn	Ile	Gln	
	305					310					315					320	
50	Val	Lys	Glu	Val	Thr	Thr	Asn	Asp	Gly	Val	Thr	Thr	Ile	Ala	Asn	Asn	
					325					330					335		
55	Leu	Thr	Ser	Thr	Val	Gln	Val	Phe	Ser	Asp	Ser	Glu	Tyr	Gln	Leu	Pro	
				340					345					350			
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			355					360					365				
65	Asp	Val	Phe	Met	Ile	Pro	Gln	Tyr	Gly	Tyr	Leu	Thr	Leu	Asn	Asn	Gly	
		370					375					380					
70	Ser	Gln	Ala	Val	Gly	Arg	Ser	Ser	Phe	Tyr	Cys	Leu	Glu	Tyr	Phe	Pro	
	385					390					395					400	

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				420					425					430			
10	Arg	Leu	Met	Asn	Pro	Leu	Ile	Asp	Gln	Tyr	Leu	Tyr	Tyr	Leu	Asn	Arg	
			435					440					445				
15	Thr	Gln	Asn	Gln	Ser	Gly	Ser	Ala	Gln	Asn	Lys	Asp	Leu	Leu	Phe	Ser	
		450					455					460					
20	Arg	Gly	Ser	Pro	Ala	Gly	Met	Ser	Val	Gln	Pro	Lys	Asn	Trp	Leu	Pro	
	465					470					475					480	
25	Gly	Pro	Cys	Tyr	Arg	Gln	Gln	Arg	Val	Ser	Lys	Thr	Lys	Thr	Asp	Asn	
					485					490					495		
30	Asn	Asn	Ser	Asn	Phe	Thr	Trp	Thr	Gly	Ala	Ser	Lys	Tyr	Asn	Leu	Asn	
				500					505					510			
35	Gly	Arg	Glu	Ser	Ile	Ile	Asn	Pro	Gly	Thr	Ala	Met	Ala	Ser	His	Lys	
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40	Asp	Asp	Glu	Asp	Lys	Phe	Phe	Pro	Met	Ser	Gly	Val	Met	Ile	Phe	Gly	
		530					535					540					
45	Lys	Glu	Ser	Ala	Gly	Ala	Ser	Asn	Thr	Ala	Leu	Asp	Asn	Val	Met	Ile	
	545					550					555					560	
50	Thr	Asp	Glu	Glu	Glu	Ile	Lys	Ala	Thr	Asn	Pro	Val	Ala	Thr	Glu	Arg	
					565				570						575		
55	Phe	Gly	Thr	Val	Ala	Val	Asn	Phe	Gln	Ser	Ser	Ser	Thr	Asp	Pro	Ala	
				580					585					590			
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65	Asp	Arg	Asp	Val	Tyr	Leu	Gln	Gly	Pro	Ile	Trp	Ala	Lys	Ile	Pro	His	
		610					615					620					

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	Thr	Asp	Gly	His	Phe	His	Pro	Ser	Pro	Leu	Met	Gly	Gly	Phe	Gly	Leu
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5	Lys	Asn	Pro	Pro	Pro	Gln	Ile	Leu	Ile	Lys	Asn	Thr	Pro	Val	Pro	Ala
					645					650					655	
10	Asn	Pro	Pro	Ala	Glu	Phe	Ser	Ala	Thr	Lys	Phe	Ala	Ser	Phe	Ile	Thr
				660					665					670		
15	Gln	Tyr	Ser	Thr	Gly	Gln	Val	Ser	Val	Glu	Ile	Glu	Trp	Glu	Leu	Gln
			675					680					685			
20	Lys	Glu	Asn	Ser	Lys	Arg	Trp	Asn	Pro	Glu	Val	Gln	Tyr	Thr	Ser	Asn
		690					695					700				
25	Tyr	Ala	Lys	Ser	Ala	Asn	Val	Asp	Phe	Thr	Val	Asp	Asn	Asn	Gly	Leu
	705					710					715					720
30	Tyr	Thr	Glu	Pro	Arg	Pro	Ile	Gly	Thr	Arg	Tyr	Leu	Thr	Arg	Pro	Leu
					725					730					735	
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				20					25					30		
45	Lys	Ala	Asn	Gln	Gln	Lys	Gln	Asp	Asp	Gly	Arg	Gly	Leu	Val	Leu	Pro
			35					40					45			
50	Gly	Tyr	Lys	Tyr	Leu	Gly	Pro	Phe	Asn	Gly	Leu	Asp	Lys	Gly	Glu	Pro
		50					55					60				
55	Val	Asn	Ala	Ala	Asp	Ala	Ala	Ala	Leu	Glu	His	Asp	Lys	Ala	Tyr	Asp
	65					70					75					80
	Gln	Gln	Leu	Gln	Ala	Gly	Asp	Asn	Pro	Tyr	Leu	Arg	Tyr	Asn	His	Ala

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	85							90							95						
5	Asp	Ala	Glu	Phe	Gln	Glu	Arg	Leu	Gln	Glu	Asp	Thr	Ser	Phe	Gly	Gly					
				100					105					110							
10	Asn	Leu	Gly	Arg	Ala	Val	Phe	Gln	Ala	Lys	Lys	Arg	Val	Leu	Glu	Pro					
			115					120					125								
15	Leu	Gly	Leu	Val	Glu	Glu	Gly	Ala	Lys	Thr	Ala	Pro	Gly	Lys	Lys	Arg					
		130					135					140									
20	Pro	Val	Glu	Pro	Ser	Pro	Gln	Arg	Ser	Pro	Asp	Ser	Ser	Thr	Gly	Ile					
						150					155					160					
25	Gly	Lys	Lys	Gly	Gln	Gln	Pro	Ala	Arg	Lys	Arg	Leu	Asn	Phe	Gly	Gln					
					165					170					175						
30	Thr	Gly	Asp	Ser	Glu	Ser	Val	Pro	Asp	Pro	Gln	Pro	Leu	Gly	Glu	Pro					
				180					185					190							
35	Pro	Ala	Ala	Pro	Ser	Gly	Val	Gly	Pro	Asn	Thr	Met	Ala	Ala	Gly	Gly					
			195					200					205								
40	Gly	Ala	Pro	Met	Ala	Asp	Asn	Asn	Glu	Gly	Ala	Asp	Gly	Val	Gly	Ser					
		210					215					220									
45	Ser	Ser	Gly	Asn	Trp	His	Cys	Asp	Ser	Thr	Trp	Leu	Gly	Asp	Arg	Val					
		225				230					235					240					
50	Ile	Thr	Thr	Ser	Thr	Arg	Thr	Trp	Ala	Leu	Pro	Thr	Tyr	Asn	Asn	His					
					245					250					255						
55	Leu	Tyr	Lys	Gln	Ile	Ser	Asn	Gly	Thr	Ser	Gly	Gly	Ala	Thr	Asn	Asp					
				260					265					270							
60	Asn	Thr	Tyr	Phe	Gly	Tyr	Ser	Thr	Pro	Trp	Gly	Tyr	Phe	Asp	Phe	Asn					
			275					280					285								
65	Arg	Phe	His	Cys	His	Phe	Ser	Pro	Arg	Asp	Trp	Gln	Arg	Leu	Ile	Asn					
		290					295					300									
70	Asn	Asn	Trp	Gly	Phe	Arg	Pro	Lys	Arg	Leu	Ser	Phe	Lys	Leu	Phe	Asn					
		305				310					315					320					

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	Ile	Gln	Val	Lys	Glu	Val	Thr	Gln	Asn	Glu	Gly	Thr	Lys	Thr	Ile	Ala
					325					330					335	
5	Asn	Asn	Leu	Thr	Ser	Thr	Ile	Gln	Val	Phe	Thr	Asp	Ser	Glu	Tyr	Gln
				340					345					350		
10	Leu	Pro	Tyr	Val	Leu	Gly	Ser	Ala	His	Gln	Gly	Cys	Leu	Pro	Pro	Phe
			355					360					365			
15	Pro	Ala	Asp	Val	Phe	Met	Ile	Pro	Gln	Tyr	Gly	Tyr	Leu	Thr	Leu	Asn
		370					375					380				
20	Asn	Gly	Ser	Gln	Ala	Val	Gly	Arg	Ser	Ser	Phe	Tyr	Cys	Leu	Glu	Tyr
	385					390					395					400
25	Phe	Pro	Ser	Gln	Met	Leu	Arg	Thr	Gly	Asn	Asn	Phe	Gln	Phe	Thr	Tyr
					405					410					415	
30	Thr	Phe	Glu	Asp	Val	Pro	Phe	His	Ser	Ser	Tyr	Ala	His	Ser	Gln	Ser
				420					425					430		
35	Leu	Asp	Arg	Leu	Met	Asn	Pro	Leu	Ile	Asp	Gln	Tyr	Leu	Tyr	Tyr	Leu
		435						440					445			
40	Ser	Arg	Thr	Gln	Thr	Thr	Gly	Gly	Thr	Ala	Asn	Thr	Gln	Thr	Leu	Gly
	450						455					460				
45	Phe	Ser	Gln	Gly	Gly	Pro	Asn	Thr	Met	Ala	Asn	Gln	Ala	Lys	Asn	Trp
	465					470					475					480
50	Leu	Pro	Gly	Pro	Cys	Tyr	Arg	Gln	Gln	Arg	Val	Ser	Thr	Thr	Thr	Gly
					485					490					495	
55	Gln	Asn	Asn	Asn	Ser	Asn	Phe	Ala	Trp	Thr	Ala	Gly	Thr	Lys	Tyr	His
				500					505					510		
60	Leu	Asn	Gly	Arg	Asn	Ser	Leu	Ala	Asn	Pro	Gly	Ile	Ala	Met	Ala	Thr
			515					520					525			
65	His	Lys	Asp	Asp	Glu	Glu	Arg	Phe	Phe	Pro	Ser	Asn	Gly	Ile	Leu	Ile
		530					535					540				

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	Phe	Gly	Lys	Gln	Asn	Ala	Ala	Arg	Asp	Asn	Ala	Asp	Tyr	Ser	Asp	Val
	545					550					555					560
5	Met	Leu	Thr	Ser	Glu	Glu	Glu	Ile	Lys	Thr	Thr	Asn	Pro	Val	Ala	Thr
					565					570					575	
10	Glu	Glu	Tyr	Gly	Ile	Val	Ala	Asp	Asn	Leu	Gln	Gln	Gln	Asn	Thr	Ala
				580					585					590		
15	Pro	Gln	Ile	Gly	Thr	Val	Asn	Ser	Gln	Gly	Ala	Leu	Pro	Gly	Met	Val
				595				600					605			
20	Trp	Gln	Asn	Arg	Asp	Val	Tyr	Leu	Gln	Gly	Pro	Ile	Trp	Ala	Lys	Ile
	610						615					620				
25	Pro	His	Thr	Asp	Gly	Asn	Phe	His	Pro	Ser	Pro	Leu	Met	Gly	Gly	Phe
	625					630					635					640
30	Gly	Leu	Lys	His	Pro	Pro	Pro	Gln	Ile	Leu	Ile	Lys	Asn	Thr	Pro	Val
					645					650					655	
35	Pro	Ala	Asn	Pro	Ser	Thr	Thr	Phe	Ser	Ala	Ala	Lys	Phe	Ala	Ser	Phe
				660					665					670		
40	Ile	Thr	Gln	Tyr	Ser	Thr	Gly	Gln	Val	Ser	Val	Glu	Ile	Glu	Trp	Glu
			675					680					685			
45	Leu	Gln	Lys	Glu	Asn	Ser	Lys	Arg	Trp	Asn	Pro	Glu	Ile	Gln	Tyr	Thr
	690						695					700				
50	Ser	Asn	Tyr	Tyr	Lys	Ser	Thr	Ser	Val	Asp	Phe	Ala	Val	Asn	Thr	Glu
	705					710					715					720
55	Gly	Val	Tyr	Ser	Glu	Pro	Arg	Pro	Ile	Gly	Thr	Arg	Tyr	Leu	Thr	Arg
					725					730					735	
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5	Gly	Val	Arg	Glu	Trp	Trp	Ala	Leu	Gln	Pro	Gly	Ala	Pro	Lys	Pro	Lys
				20					25					30		
	Ala	Asn	Gln	Gln	His	Gln	Asp	Asn	Ala	Arg	Gly	Leu	Val	Leu	Pro	Gly
10			35					40					45			
	Tyr	Lys	Tyr	Leu	Gly	Pro	Gly	Asn	Gly	Leu	Asp	Lys	Gly	Glu	Pro	Val
		50					55					60				
15																
	Asn	Ala	Ala	Asp	Ala	Ala	Ala	Leu	Glu	His	Asp	Lys	Ala	Tyr	Asp	Gln
	65					70					75					80
20	Gln	Leu	Lys	Ala	Gly	Asp	Asn	Pro	Tyr	Leu	Lys	Tyr	Asn	His	Ala	Asp
					85					90					95	
	Ala	Glu	Phe	Gln	Gln	Arg	Leu	Gln	Gly	Asp	Thr	Ser	Phe	Gly	Gly	Asn
25				100					105					110		
	Leu	Gly	Arg	Ala	Val	Phe	Gln	Ala	Lys	Lys	Arg	Val	Leu	Glu	Pro	Leu
			115					120					125			
30																
	Gly	Leu	Val	Glu	Gln	Ala	Gly	Glu	Thr	Ala	Pro	Gly	Lys	Lys	Arg	Pro
		130					135					140				
35	Leu	Ile	Glu	Ser	Pro	Gln	Gln	Pro	Asp	Ser	Ser	Thr	Gly	Ile	Gly	Lys
	145					150					155					160
	Lys	Gly	Lys	Gln	Pro	Ala	Lys	Lys	Lys	Leu	Val	Phe	Glu	Asp	Glu	Thr
40					165					170					175	
	Gly	Ala	Gly	Asp	Gly	Pro	Pro	Glu	Gly	Ser	Thr	Ser	Gly	Ala	Met	Ser
				180					185					190		
45																
	Asp	Asp	Ser	Glu	Met	Arg	Ala	Ala	Ala	Gly	Gly	Ala	Ala	Val	Glu	Gly
			195					200					205			
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		210					215					220				
55																

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	Asp	Ser	Thr	Trp	Ser	Glu	Gly	His	Val	Thr	Thr	Thr	Ser	Thr	Arg	Thr
	225					230					235					240
5	Trp	Val	Leu	Pro	Thr	Tyr	Asn	Asn	His	Leu	Tyr	Lys	Arg	Leu	Gly	Glu
					245					250					255	
10	Ser	Leu	Gln	Ser	Asn	Thr	Tyr	Asn	Gly	Phe	Ser	Thr	Pro	Trp	Gly	Tyr
				260					265					270		
15	Phe	Asp	Phe	Asn	Arg	Phe	His	Cys	His	Phe	Ser	Pro	Arg	Asp	Trp	Gln
			275					280					285			
20	Arg	Leu	Ile	Asn	Asn	Asn	Trp	Gly	Met	Arg	Pro	Lys	Ala	Met	Arg	Val
	290						295					300				
25	Lys	Ile	Phe	Asn	Ile	Gln	Val	Lys	Glu	Val	Thr	Thr	Ser	Asn	Gly	Glu
	305					310					315					320
30	Thr	Thr	Val	Ala	Asn	Asn	Leu	Thr	Ser	Thr	Val	Gln	Ile	Phe	Ala	Asp
					325					330					335	
35	Ser	Ser	Tyr	Glu	Leu	Pro	Tyr	Val	Met	Asp	Ala	Gly	Gln	Glu	Gly	Ser
				340					345					350		
40	Leu	Pro	Pro	Phe	Pro	Asn	Asp	Val	Phe	Met	Val	Pro	Gln	Tyr	Gly	Tyr
			355					360					365			
45	Cys	Gly	Leu	Val	Thr	Gly	Asn	Thr	Ser	Gln	Gln	Gln	Thr	Asp	Arg	Asn
	370						375					380				
50	Ala	Phe	Tyr	Cys	Leu	Glu	Tyr	Phe	Pro	Ser	Gln	Met	Leu	Arg	Thr	Gly
	385					390					395					400
55	Asn	Asn	Phe	Glu	Ile	Thr	Tyr	Ser	Phe	Glu	Lys	Val	Pro	Phe	His	Ser
				405						410					415	
60	Met	Tyr	Ala	His	Ser	Gln	Ser	Leu	Asp	Arg	Leu	Met	Asn	Pro	Leu	Ile
				420					425					430		
65	Asp	Gln	Tyr	Leu	Trp	Gly	Leu	Gln	Ser	Thr	Thr	Thr	Thr	Thr	Leu	Asn
			435					440					445			

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	Ala	Gly	Thr	Ala	Thr	Thr	Asn	Phe	Thr	Lys	Leu	Arg	Pro	Thr	Asn	Phe
	450						455					460				
5	Ser	Asn	Phe	Lys	Lys	Asn	Trp	Leu	Pro	Gly	Pro	Ser	Ile	Lys	Gln	Gln
	465					470					475					480
10	Gly	Phe	Ser	Lys	Thr	Ala	Asn	Gln	Asn	Tyr	Lys	Ile	Pro	Ala	Thr	Gly
					485					490					495	
15	Ser	Asp	Ser	Leu	Ile	Lys	Tyr	Glu	Thr	His	Ser	Thr	Leu	Asp	Gly	Arg
				500					505					510		
20	Trp	Ser	Ala	Leu	Thr	Pro	Gly	Pro	Pro	Met	Ala	Thr	Ala	Gly	Pro	Ala
			515					520					525			
25	Asp	Ser	Lys	Phe	Ser	Asn	Ser	Gln	Leu	Ile	Phe	Ala	Gly	Pro	Lys	Gln
	530						535					540				
30	Asn	Gly	Asn	Thr	Ala	Thr	Val	Pro	Gly	Thr	Leu	Ile	Phe	Thr	Ser	Glu
	545					550					555					560
35	Glu	Glu	Leu	Ala	Ala	Thr	Asn	Ala	Thr	Asp	Thr	Asp	Met	Trp	Gly	Asn
					565					570					575	
40	Leu	Pro	Gly	Gly	Asp	Gln	Ser	Asn	Ser	Asn	Leu	Pro	Thr	Val	Asp	Arg
				580					585					590		
45	Leu	Thr	Ala	Leu	Gly	Ala	Val	Pro	Gly	Met	Val	Trp	Gln	Asn	Arg	Asp
			595					600					605			
50	Ile	Tyr	Tyr	Gln	Gly	Pro	Ile	Trp	Ala	Lys	Ile	Pro	His	Thr	Asp	Gly
	610						615					620				
55	His	Phe	His	Pro	Ser	Pro	Leu	Ile	Gly	Gly	Phe	Gly	Leu	Lys	His	Pro
	625					630					635					640
60	Pro	Pro	Gln	Ile	Phe	Ile	Lys	Asn	Thr	Pro	Val	Pro	Ala	Asn	Pro	Ala
					645					650					655	
65	Thr	Thr	Phe	Ser	Ser	Thr	Pro	Val	Asn	Ser	Phe	Ile	Thr	Gln	Tyr	Ser
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	Thr Gly Gln Val Ser Val Gln Ile Asp Trp Glu Ile Gln Lys Glu Arg	
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5	Ser Lys Arg Trp Asn Pro Glu Val Gln Phe Thr Ser Asn Tyr Gly Gln	
	690	700
10	Gln Asn Ser Leu Leu Trp Ala Pro Asp Ala Ala Gly Lys Tyr Thr Glu	
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		715
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	gctcgaggtc ttgtgcttcc tgggtacaag tatcttggtc ctgggaacgg ccttgataag	180
50	ggcgatcctg tcaattttgc tgacgagggt gcccgagagc acgacctctc ctaccagaaa	240
	cagcttgagg cgggcgataa cccttacctc aagtacaacc acgcggacgc agagtttcag	300
55	gagaaactcg cttctgacac ttcttttggg ggaaccttg ggaaggctgt tttccaggct	360

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5	aagaaaggca	aacagcctgc	cagaaagaga	ctcaactttg	acgacgaacc	tggagccgga	540
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	gcagcagctg	gcggaaatgg	tggcgatgcg	ggacaagggtg	ccgagggagt	gggtaatgcc	660
10	tccggtgatt	ggcattgcga	ttccacttgg	tcagagagcc	acgtcaccac	cacctcaacc	720
	cgcacctggg	tcttgccgac	ctacaacaac	cacctgtacc	tgcggctcgg	ctcgagcaac	780
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20	ggggagacga	ccgtatccaa	caacctcacc	agcacggtcc	atatctttgc	ggacagcacg	1020
	tacgagctcc	cgtacgtgat	ggatgcaggt	caggagggca	gcttgccctcc	tttccccaac	1080
	gacgtgttca	tgggtgcctca	gtacgggtac	tgcggactgg	taaccggagg	cagctctcaa	1140
25	aaccagacag	acagaaatgc	cttctactgt	ctggagtact	ttcccagcca	gatgctgaga	1200
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50	aaaagcccgc	ctccacaaat	attcatcaaa	aacactcctg	taccgcgcaa	tcccgcgaacg	1980
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20

Ala Asn Gln Gln Lys Gln Asp Asn Ala Arg Gly Leu Val Leu Pro Gly
 35 40 45

25

Tyr Lys Tyr Leu Gly Pro Gly Asn Gly Leu Asp Lys Gly Asp Pro Val
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Asn Phe Ala Asp Glu Val Ala Arg Glu His Asp Leu Ser Tyr Gln Lys
 65 70 75 80

30

Gln Leu Glu Ala Gly Asp Asn Pro Tyr Leu Lys Tyr Asn His Ala Asp
 85 90 95

Ala Glu Phe Gln Glu Lys Leu Ala Ser Asp Thr Ser Phe Gly Gly Asn
 100 105 110

35

Leu Gly Lys Ala Val Phe Gln Ala Lys Lys Arg Ile Leu Glu Pro Leu
 115 120 125

40

Gly Leu Val Glu Thr Pro Asp Lys Thr Ala Pro Ala Ala Lys Lys Arg
 130 135 140

45

Pro Leu Glu Gln Ser Pro Gln Glu Pro Asp Ser Ser Ser Gly Val Gly
 145 150 155 160

50

Lys Lys Gly Lys Gln Pro Ala Arg Lys Arg Leu Asn Phe Asp Asp Glu
 165 170 175

55

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10	Asp	Ala	Gly	Gln	Gly	Ala	Glu	Gly	Val	Gly	Asn	Ala	Ser	Gly	Asp	Trp
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15	His	Cys	Asp	Ser	Thr	Trp	Ser	Glu	Ser	His	Val	Thr	Thr	Thr	Ser	Thr
	225					230					235					240
20	Arg	Thr	Trp	Val	Leu	Pro	Thr	Tyr	Asn	Asn	His	Leu	Tyr	Leu	Arg	Leu
				245						250					255	
25	Gly	Ser	Ser	Asn	Ala	Ser	Asp	Thr	Phe	Asn	Gly	Phe	Ser	Thr	Pro	Trp
				260					265					270		
30	Gly	Tyr	Phe	Asp	Phe	Asn	Arg	Phe	His	Cys	His	Phe	Ser	Pro	Arg	Asp
			275					280					285			
35	Trp	Gln	Arg	Leu	Ile	Asn	Asn	His	Trp	Gly	Leu	Arg	Pro	Lys	Ser	Met
		290					295					300				
40	Gln	Val	Arg	Ile	Phe	Asn	Ile	Gln	Val	Lys	Glu	Val	Thr	Thr	Ser	Asn
	305					310					315					320
45	Gly	Glu	Thr	Thr	Val	Ser	Asn	Asn	Leu	Thr	Ser	Thr	Val	His	Ile	Phe
					325					330					335	
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				340					345					350		
55	Gly	Ser	Leu	Pro	Pro	Phe	Pro	Asn	Asp	Val	Phe	Met	Val	Pro	Gln	Tyr
			355					360					365			
60	Gly	Tyr	Cys	Gly	Leu	Val	Thr	Gly	Gly	Ser	Ser	Gln	Asn	Gln	Thr	Asp
		370					375					380				
65	Arg	Asn	Ala	Phe	Tyr	Cys	Leu	Glu	Tyr	Phe	Pro	Ser	Gln	Met	Leu	Arg
	385					390					395					400

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	Thr	Gly	Asn	Asn	Phe	Glu	Met	Val	Tyr	Lys	Phe	Glu	Asn	Val	Pro	Phe	
					405					410					415		
5	His	Ser	Met	Tyr	Ala	His	Ser	Gln	Ser	Leu	Asp	Arg	Leu	Met	Asn	Pro	
				420					425					430			
10	Leu	Leu	Asp	Gln	Tyr	Leu	Trp	Glu	Leu	Gln	Ser	Thr	Thr	Ser	Gly	Gly	
			435					440					445				
15	Thr	Leu	Asn	Gln	Gly	Asn	Ser	Ala	Thr	Asn	Phe	Ala	Lys	Leu	Thr	Asn	
		450					455					460					
20	Lys	Asn	Phe	Ser	Gly	Tyr	Arg	Lys	Asn	Trp	Leu	Pro	Gly	Pro	Met	Met	
	465					470					475					480	
25	Lys	Gln	Gln	Arg	Phe	Ser	Lys	Thr	Ala	Ser	Gln	Asn	Tyr	Lys	Ile	Pro	
					485					490					495		
30	Gln	Gly	Gly	Asn	Asn	Ser	Leu	Leu	His	Tyr	Glu	Thr	Arg	Thr	Thr	Leu	
				500					505					510			
35	Asp	Arg	Arg	Trp	Ser	Asn	Phe	Ala	Pro	Gly	Thr	Ala	Met	Ala	Thr	Ala	
			515					520					525				
40	Ala	Asn	Asp	Ala	Thr	Asp	Phe	Ser	Gln	Ala	Gln	Leu	Ile	Phe	Ala	Gly	
		530					535					540					
45	Pro	Asn	Ile	Thr	Gly	Asn	Thr	Thr	Thr	Asp	Ala	Asn	Asn	Leu	Met	Phe	
	545					550					555					560	
50	Thr	Ser	Glu	Asp	Glu	Leu	Arg	Ala	Thr	Asn	Pro	Arg	Asp	Thr	Asp	Leu	
					565					570					575		
55	Phe	Gly	His	Leu	Ala	Thr	Asn	Gln	Gln	Asn	Ala	Thr	Thr	Val	Pro	Thr	
				580					585					590			
60	Val	Asp	Asp	Val	Asp	Gly	Val	Gly	Val	Tyr	Pro	Gly	Met	Val	Trp	Gln	
			595					600					605				
65	Asp	Arg	Asp	Ile	Tyr	Tyr	Gln	Gly	Pro	Ile	Trp	Ala	Lys	Ile	Pro	His	
		610					615					620					
70	Thr	Asp	Gly	His	Phe	His	Pro	Ser	Pro	Leu	Ile	Gly	Gly	Phe	Gly	Leu	

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	625				630					635					640	
5	Lys	Ser	Pro	Pro	Pro	Gln	Ile	Phe	Ile	Lys	Asn	Thr	Pro	Val	Pro	Ala
					645					650					655	
10	Asn	Pro	Ala	Thr	Thr	Phe	Ser	Pro	Ala	Arg	Ile	Asn	Ser	Phe	Ile	Thr
				660					665					670		
15	Gln	Tyr	Ser	Thr	Gly	Gln	Val	Ala	Val	Lys	Ile	Glu	Trp	Glu	Ile	Gln
			675					680					685			
20	Lys	Glu	Arg	Ser	Lys	Arg	Trp	Asn	Pro	Glu	Val	Gln	Phe	Thr	Ser	Asn
		690					695					700				
25	Tyr	Gly	Ala	Gln	Asp	Ser	Leu	Leu	Trp	Ala	Pro	Asp	Asn	Ala	Gly	Ala
	705					710					715					720
30	Tyr	Lys	Glu	Pro	Arg	Ala	Ile	Gly	Ser	Arg	Tyr	Leu	Thr	Asn	His	Leu
					725					730					735	
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	<213> Artificial Sequence															
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75	<400> 29															

His Lys Asp Asp Glu Ala Lys Phe Phe Pro Gln
 1 5 10

Claims

1. Use of a mutated adeno-associated virus (AAV) capsid protein, within a recombinant AAV virion, for decreasing the immunoreactivity of the virion as compared to the corresponding wild-type virion, wherein said mutated AAV capsid protein comprises a substitution of the amino acid which occurs at the position which corresponds to position 334 of the AAV-2 VP2 capsid protein.
2. The use according to claim 1, wherein the amino acid which occurs at the position which corresponds to position 334 of the AAV-2 VP2 capsid protein is substituted with an alanine.
3. The use according to claim 1 or claim 2, wherein said recombinant AAV virion comprises a heterologous nucleic acid molecule.
4. The use of any one of claims 1 to 3, wherein said recombinant AAV virion comprises a heterologous nucleic acid molecule encoding an antisense RNA or ribozyme.
5. The use of any one of claims 1 to 4, wherein said recombinant AAV virion comprises a heterologous nucleic acid molecule encoding a therapeutic protein, operably linked to control elements capable of directing the *in vivo* transcription and translation of said therapeutic protein.
6. A recombinant adeno-associated virus (AAV) virion with decreased immunoreactivity as defined in claim 5, for use in a method of treatment of a vertebrate subject, whereby the product encoded by the heterologous nucleic acid is expressed at a level that provides a therapeutic effect and wherein the subject has been previously exposed to any of the AAV serotypes.
7. The recombinant AAV virion for use according to claim 6, wherein said cell or tissue is a muscle cell or tissue, and is preferably derived from skeletal muscle.
8. The recombinant AAV virion for use according to claim 6 or claim 7, wherein said delivery is (i) by intramuscular injection; (ii) into the bloodstream; (iii) intravenously; (iv) intraarterially; (v) to the liver; or (vi) to the brain.
9. The recombinant AAV virion for use according to any one of claims 6 to 8, wherein said delivery is (i) by intramuscular injection; (ii) into the bloodstream; or (iii) to the liver.

Patentansprüche

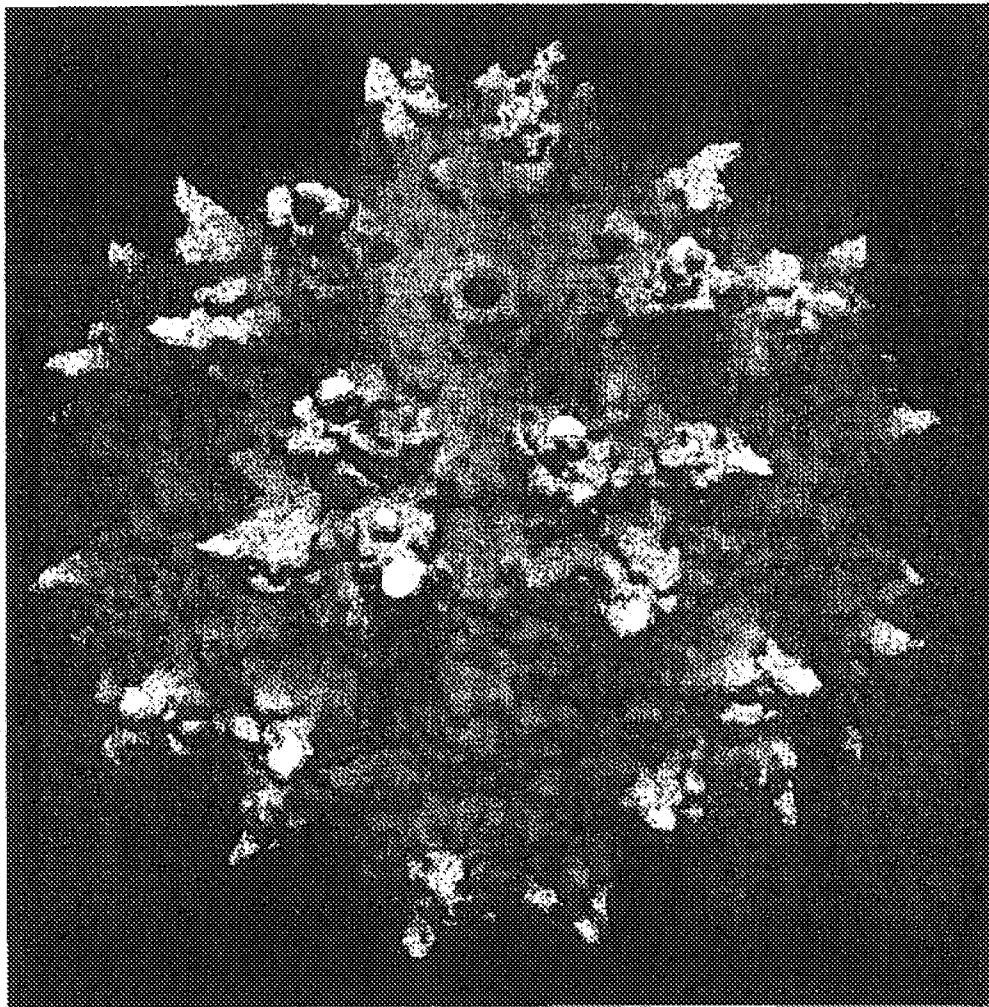
1. Verwendung eines mutierten Adeno-assoziiertes-Virus(AAV)-Capsidproteins in einem rekombinanten AAV-Virion zur Verringerung der Immunreaktivität des Virions verglichen mit dem entsprechenden Wildtypvirion, wobei das mutierte AAV-Capsid-protein eine Substitution der Aminosäure, die an der Position 334 des AAV-2-VP2-Capsid-proteins entsprechenden Position vorkommt, umfasst.
2. Verwendung gemäß Anspruch 1, wobei die Aminosäure, die an der Position 334 des AAV-2-VP2-Capsid-proteins entsprechenden Position vorkommt, durch ein Alanin substituiert ist.
3. Verwendung gemäß Anspruch 1 oder Anspruch 2, wobei das rekombinante AAV-Virion ein heterologes Nukleinsäuremolekül umfasst.
4. Verwendung nach einem der Ansprüche 1 bis 3, wobei das rekombinante AAV-Virion ein heterologes Nukleinsäuremolekül umfasst, das eine Antisense-RNA oder ein Ribozym codiert.
5. Verwendung nach einem der Ansprüche 1 bis 4, wobei das rekombinante AAV-Virion ein heterologes Nukleinsäuremolekül, das ein therapeutisches Protein codiert, in operativer Verknüpfung mit zur Steuerung der In-vivo-Transkription und -Translation des therapeutischen Proteins fähigen Kontrollelementen umfasst.

6. Rekombinantes Adeno-assoziiertes-Virus(AAV)-Virion mit verminderter Immunreaktivität mit der in Anspruch 5 angegebenen Bedeutung zur Verwendung bei einem Verfahren zur Behandlung eines Verte-braten-individuums, wodurch das von der heterologen Nukleinsäure codierte Produkt auf einem Niveau exprimiert wird, das eine therapeutische Wirkung liefert, und wobei das Individuum bereits einem der AAV-Serotypen ausgesetzt war.
7. Rekombinantes AAV-Virion zur Verwendung gemäß Anspruch 6, wobei es sich bei der Zelle bzw. dem Gewebe um eine Muskelzelle bzw. ein Muskelgewebe handelt, die bzw. das vorzugsweise von Skelett-muskel stammt.
8. Rekombinantes AAV-Virion zur Verwendung gemäß Anspruch 6 oder Anspruch 7, wobei die Zuführung (i) durch intramuskuläre Injektion; (ii) in die Blutbahn; (iii) intravenös; (iv) intraarteriell; (v) an die Leber; oder (vi) an das Gehirn erfolgt.
9. Rekombinantes AAV-Virion zur Verwendung gemäß einem der Ansprüche 6 bis 8, wobei die Zuführung (i) durch intramuskuläre Injektion; (ii) in die Blutbahn; oder (iii) an die Leber erfolgt.

Revendications

1. Utilisation d'une protéine de capsid de virus adéno-associé (AAV) mutée, dans un virion d'AAV recombinant, pour diminuer l'immunoréactivité du virion par rapport au virion de type sauvage correspondant, ladite protéine de capsid d'AAV mutée comprenant une substitution de l'acide aminé présent à la position qui correspond à la position 334 de la protéine de capsid VP2 d'AAV-2.
2. Utilisation selon la revendication 1, dans laquelle l'acide aminé présent à la position qui correspond à la position 334 de la protéine de capsid VP2 d'AAV-2 est substitué par une alanine.
3. Utilisation selon la revendication 1 ou la revendication 2, dans laquelle ledit virion d'AAV recombinant comprenant une molécule d'acide nucléique hétérologue.
4. Utilisation de l'une quelconque des revendications 1 à 3, dans laquelle ledit virion d'AAV recombinant comprend une molécule d'acide nucléique hétérologue codant pour un ARN antisens ou un ribozyme.
5. Utilisation de l'une quelconque des revendications 1 à 4, dans laquelle ledit virion d'AAV recombinant comprend une molécule d'acide nucléique hétérologue codant pour une protéine thérapeutique, fonctionnellement liée à des éléments de contrôle capables de commander la transcription et la traduction *in vivo* de ladite protéine thérapeutique.
6. Virion de virus adéno-associé (AAV) recombinant ayant une immunoréactivité réduite tel que défini dans la revendication 5, pour utilisation dans un procédé de traitement d'un sujet vertébré, où le produit codé par l'acide nucléique hétérologue est exprimé à un taux qui produit un effet thérapeutique et où le sujet a été précédemment exposé à l'un quelconque des sérotypes AAV.
7. Virion d'AAV recombinant pour utilisation selon la revendication 6, ladite cellule ou ledit tissu est une cellule ou un tissu musculaire, et est de préférence dérivé de muscle squelettique.
8. Virion d'AAV recombinant pour utilisation selon la revendication 6 ou la revendication 7, ladite administration étant effectuée (i) par injection intramusculaire ; (ii) dans la circulation sanguine ; (iii) par voie intraveineuse ; (iv) par voie intra-artérielle ; (v) dans le foie ; ou (vi) dans le cerveau.
9. Virion d'AAV recombinant pour utilisation selon l'une quelconque des revendications 6 à 8, ladite administration étant effectuée (i) par injection intramusculaire ; (ii) dans la circulation sanguine ; ou (iii) dans le foie.

FIG 1.



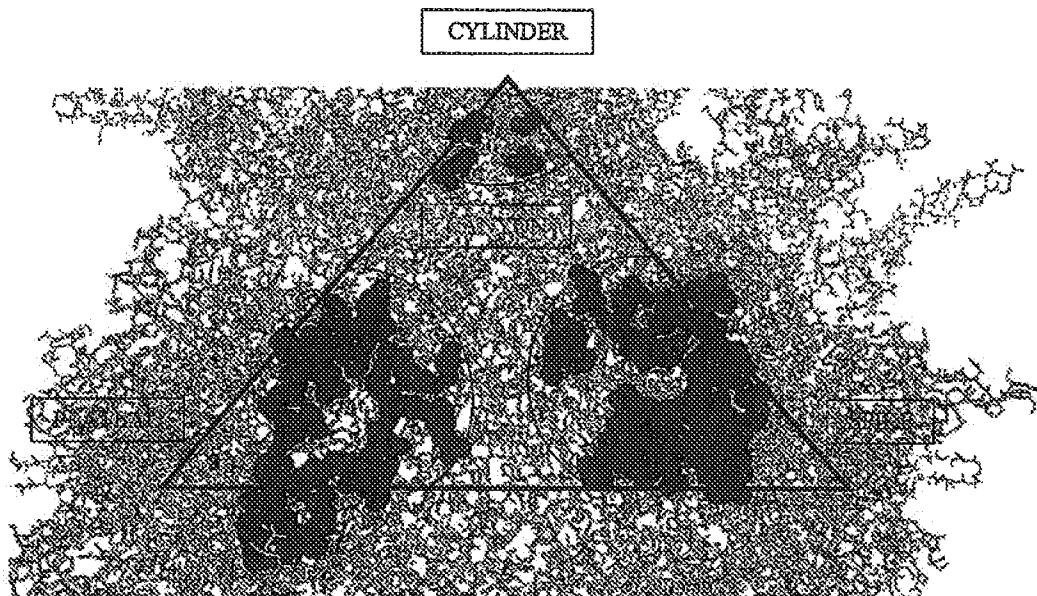


FIG. 2

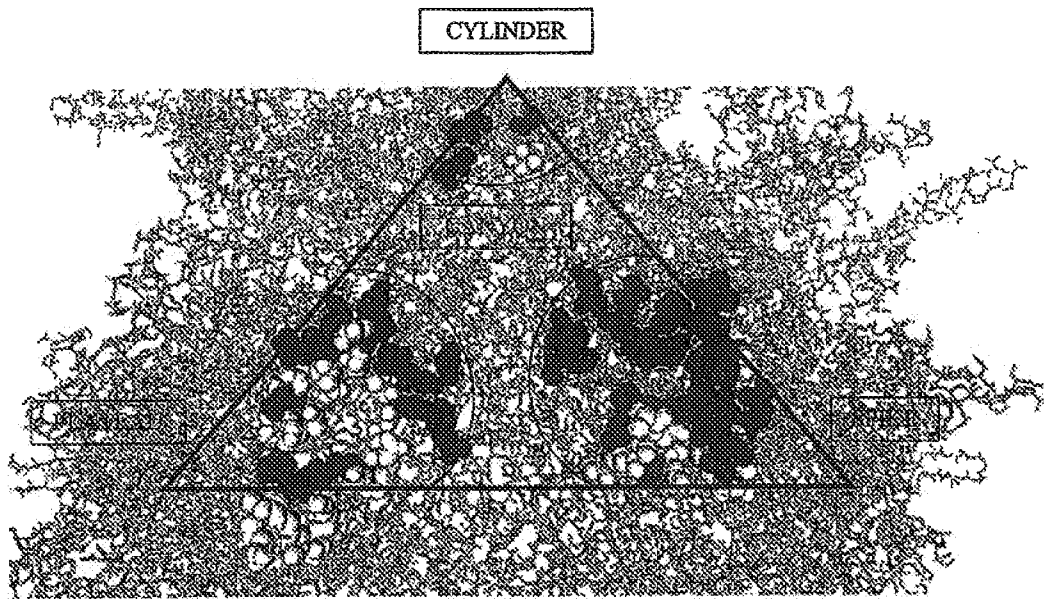


FIG. 3

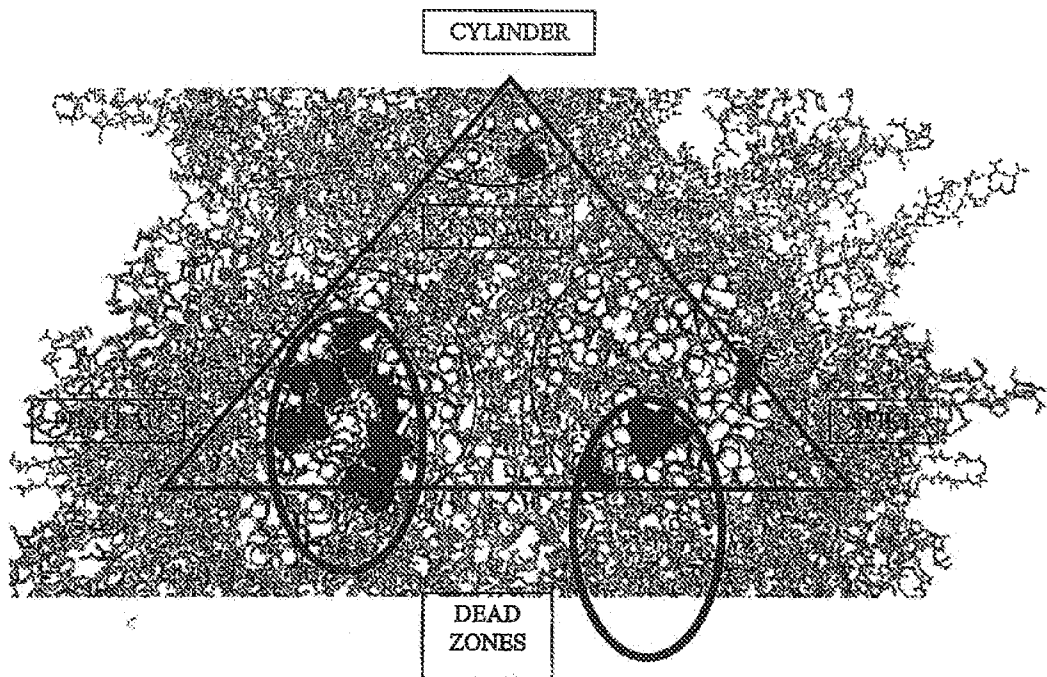


FIG. 4

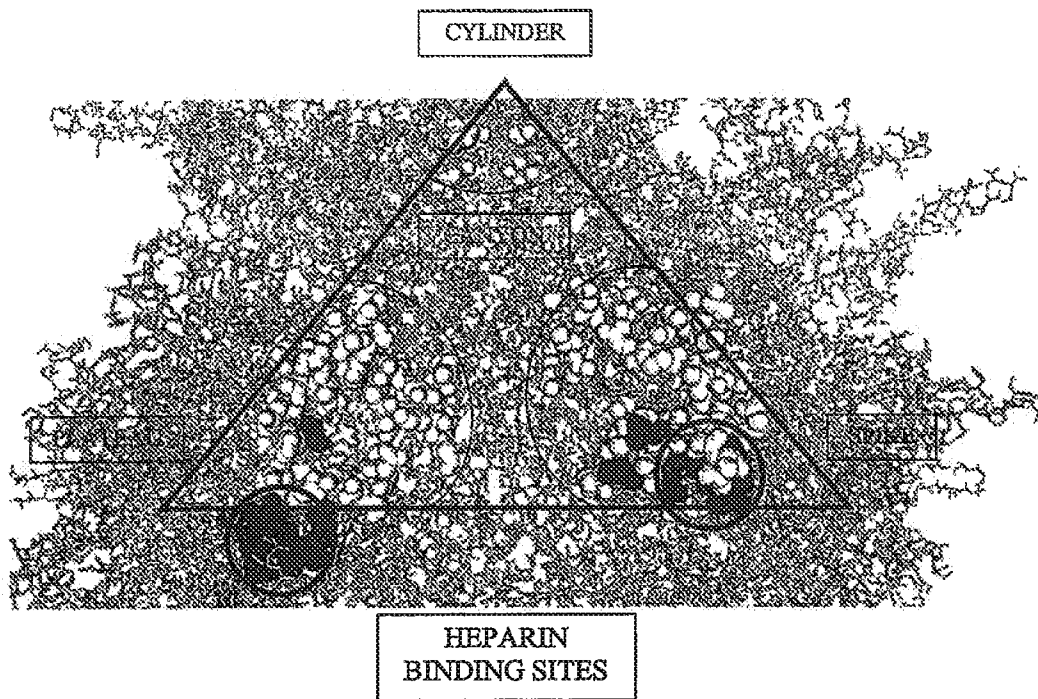


FIG. 5

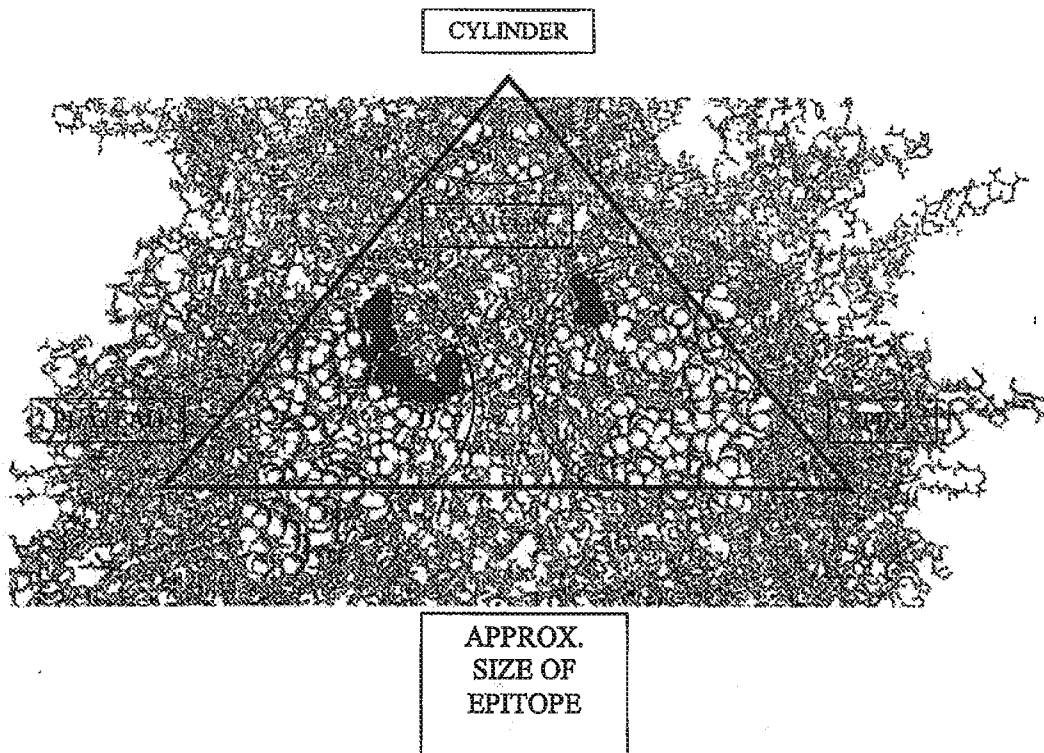


FIG. 6

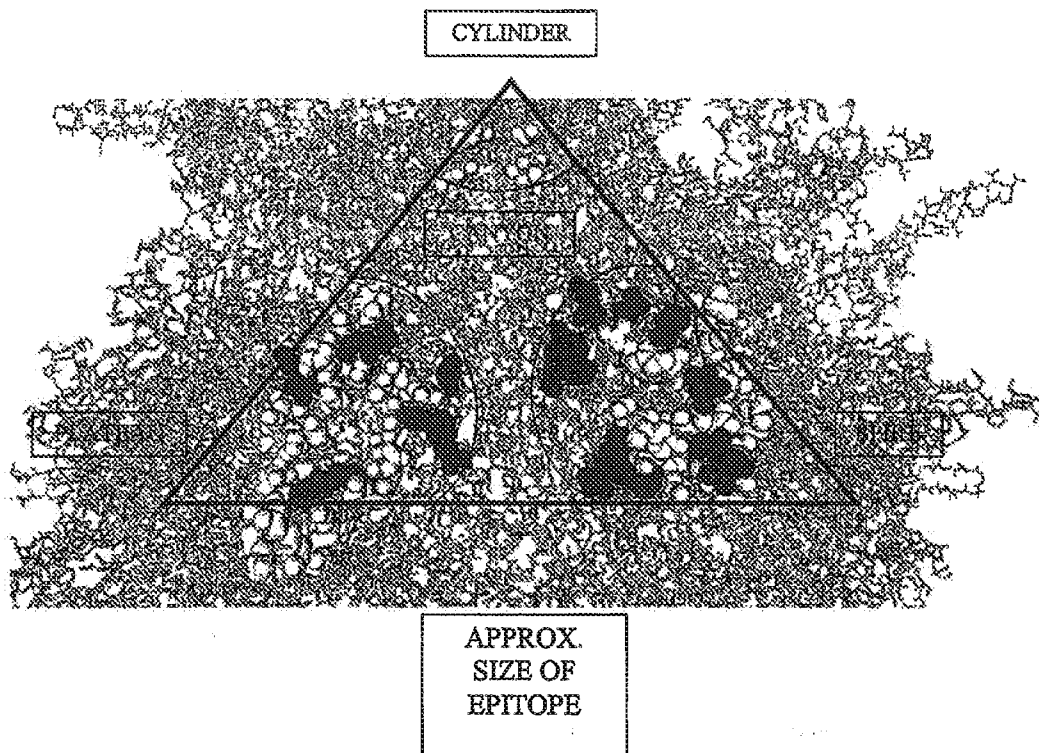
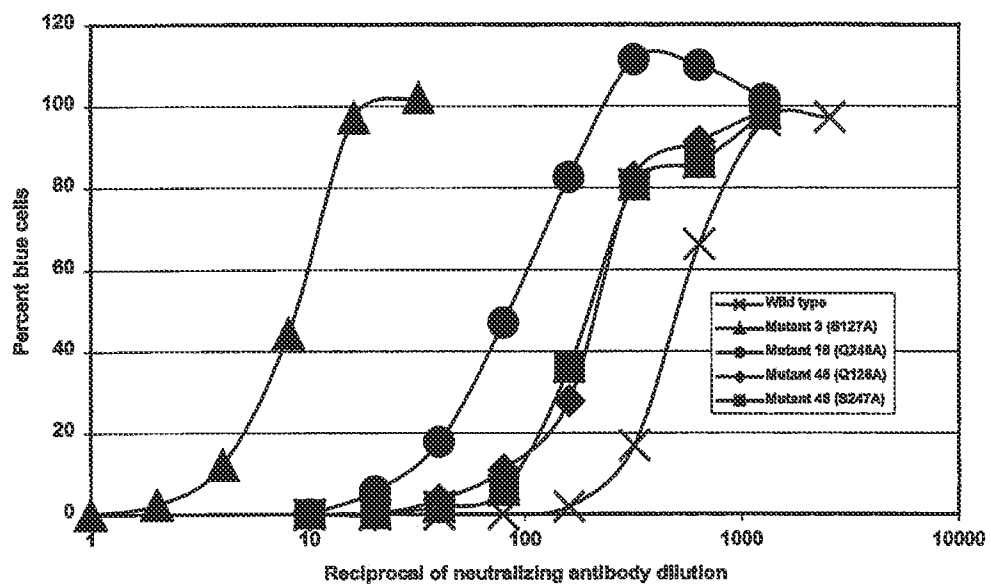


FIG. 7

FIG. 8



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1      MAPGKKRPVEHSPVEPDSSSGTGKAGQQPARKRLNFGQTGDADSVDPQPPLGQPPAAF
59     SGLGTNTMATGSGAPMADNNEGADGVGNSSGNWHCDSTWMGDRVITTTSTRTWALFTYN
117    NHLYKQISSQSGASNDNHYFGYSTPWGYFDENRFHCHFSPRDWQRLINNNWGFRPKRL
175    NEKLFNIQVKEVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSAHQGCLPFPFADV
233    FMVPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFTFSYTFEDVFFHSSYAHS
291    QSLDRLMNPLIDQYLYLSRTNTPSGTTTQSRLLQFSQAGASDIRDQSRNWLPGPCYRQ
349    QRVSKTSADNNNSEYSWTGATKYHLNGRDSLVPNGPAMASHKDDEEKFFPQSGVLIFG
407    KQGSEKTNVDIEKVMITDEEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVNTQGVL
465    PGMVWQDRDVYLQGPWAKIEHTDGHFHPSPLMGGFGLKHPPPPQILIKNTFVPANEST
523    TFSAAKFASFITQYSTGQVSVEIEWELQENSKRWNPFIQYTSNYNKSVNVDFTVDTN
581    GUYSEPRPIGTRYLTRNL

```

FIGURE 9

```

1  maadgylpdw ledtlsegir qwwklkpgpp ppkpaerhkd dsrglvpjgy kyigpfnfld
61 kgepvneada aalehdkeyd rqldegdmpy lkynhadaef qarlkedtsf ggnlgravfq
121 akkrvleplg lveepvktap gkkpvehsp vepdsssgtg kagqgparkr lnfggtgdad
181 svdpqpplgq ppaapsqigt ntmatgsgap madnnegadg vgnssgnwhc dstwmqdrvi
241 ttstrtwalp tynnhlykqi ssqsgasndn hyfgystpwg yfdfnrxfch fsprdwqrli
301 nnnwgfrpkr lnfklniqv kevtqndgtt tiannltstv qvftdseyql pyvlgshqg
361 clppfpadvf mvpqygyltl nngsqavgrs sfycleyfps qmlrtgmnft fsytfedvpf
421 hssyahsqsl drlmpilidq yllylartnt pagtttqsrl qfsqagasdi rdqsrnlwlp
481 pcyrqgrvsk tsadnnunsey swtgatkyhl ngrdsilvnpq pamashkdde ekffpqsgvl
541 ifgkqgsakt nvdiekvmit deesirtnp vataqygsvs tnlqrqnraq atadvntqgv
601 lpgmvwqdrd vylqgpiwak iphtdghfhp splmggfglk hpppqililk tppvpanpsett
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721 seprpigtry ltrnl

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FIGURE 10

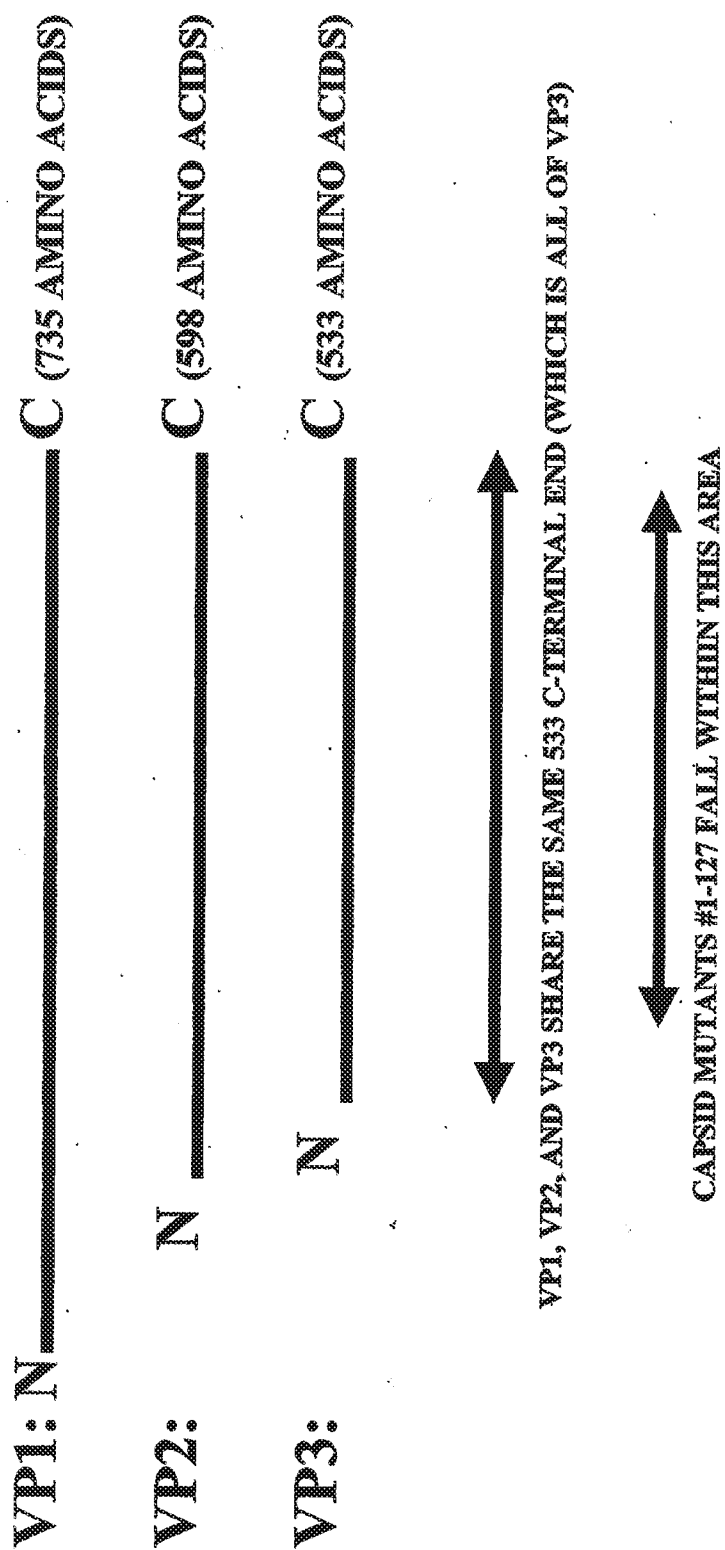


FIGURE 11

		VF1	
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Caprine AAV			
Primate AAV type 5		aacccaatcagcagcatcaagatcaagcccggtggtcttgtgctgcctggt	2350
Caprine AAV			
Primate AAV type 5		tataactatctcggaccggaaacggtctcgatcgaggagagcctgtcaa	2400
Caprine AAV			
Primate AAV type 5		cagggcagacgaggtcgcgcgagagcacgacatctcgtacaacgagcagc	2450
Caprine AAV			
Primate AAV type 5		ttgaggcgggagacaacccctacctcaagtacaaccacgcgacgcccag	2500
Caprine AAV			
Primate AAV type 5		tttcaggagaagctcgcgcgacgacacatccttcgggggaaacctcgaaa	2550
Caprine AAV			
Primate AAV type 5		ggcagtccttcaggccaagaaagggttctcgaaacctttggcctggttg	2600
Caprine AAV			
Primate AAV type 5		aagagggtgctaagacggccctaccggaaagcggatagacgaccacttt	2650
Caprine AAV			
Primate AAV type 5		ccaaaaagaaagaggctcggaccgaagaggactccaagccttccacctc	2700
Caprine AAV			
Primate AAV type 5		gtcagacgcgaagctggaccacggatcccagcagctgcaaatcccag	2750
Caprine AAV			
Primate AAV type 5		cccaaccagcctcaagtttgggagctgatacaatgtctgcgggaggtggc	2800
Caprine AAV		.a.....	
Primate AAV type 5		ggcccatctgggcgacaataaccaaggtgccgatggagtgggcaatgcctc	2850
Caprine AAV			
Primate AAV type 5		gggagattggcattgcgattccacgtggatgggggacagagtcgtcacca	2900
Caprine AAV			
Primate AAV type 5		agtccaccgcaacctgggtgctgccagctacaacaaccaccagtaccga	2950
Caprine AAV		C.....	
Primate AAV type 5		gagatcaaaagcggctccgtcgacggaagcaacgccaacgcctactttgg	3000
Caprine AAV			
Primate AAV type 5		atacagcaccctctgggggtactttgactttaaccgcttccacagccact	3050
Caprine AAV			
Primate AAV type 5		ggagcccccgagactggcaagactcatcaacaactactggggttcaga	3100
Caprine AAV		t.....	
Primate AAV type 5		ccccggtccctcagagtcaaaatcttcaacattcaagtcaaagaggtcac	3150
Caprine AAV		t.....C.....	
Primate AAV type 5		ggtgcaggactccaccaccacatcgccaacaacctcacctccaccgtcc	3200
Caprine AAV			
Primate AAV type 5		aagtgtttacggacgacgactaccagctgccctacgtcgtcggaacggg	3250
Caprine AAV		A..C..g.....	
Primate AAV type 5		accgagggatgcctgcggccttccctccgcaggtctttacgtgcgcga	3300
Caprine AAV		C.....	

FIGURE 12A

Primate AAV type 5	gtacggttacgcgacgctgaaccgcgacacacagaaaatcccaccgaga	3350
Caprine AAV	C.....a.....gg...C...C...g...a...C	
Primate AAV type 5	ggagcagcttcttctgcctagagtactttcccagcaagatgctgagaacg	3400
Caprine AAV	t.....g...	
Primate AAV type 5	ggcaacaactttgagtttacctacaactttgaggaggtgcccttccactc	3450
Caprine AAV	g.....a.....g	
Primate AAV type 5	cagcttcgctcccagtcagaacctgttcaagctggccaacccgctgggtg	3500
Caprine AAV	C...g...C.....C...t.....	
Primate AAV type 5	accagtaactgttacccgcttcgtgagcacaataaactggcggagtcacg	3550
Caprine AAV	C.....ctcggc...g...cca.....	
Primate AAV type 5	ttcaacaagaacctggccgggagatacgccaacacctacaaaaactggtt	3600
Caprine AAV	...C.a.....g...C.....	
Primate AAV type 5	ccccggggcccatgggcccgaacccagggtggaaacctgggctccggggtca	3650
Caprine AAV	ac.a...t., ..	
Primate AAV type 5	accgcgc---cagtgtcagcgccttcgccacgacc-----aataggatg	3691
Caprine AAV	g.a..a.caa...a.....t.aa.aa.tttt..gtctca..cc.....	
Primate AAV type 5	gagctcgagggcgaggtaccaggtgcccccgagccgaacggcatgac	3741
Caprine AAV	a.C..g....g..C..C....a...aa...C....C....g....	
Primate AAV type 5	caacaacctccagggcagcaacacctatgccctggagaacactatgatct	3791
Caprine AAV	a....Cg.....a.....Cg...C..g....a....C.....	
Primate AAV type 5	tcaacagccagccggcgaaacccgggcaccacccgccaactacctcgagggc	3841
Caprine AAV	gct..aaac..C.cg....a..t...t.ggt....ca....a.	
Primate AAV type 5	aacatgctcatcaccagcgagagcgagacgcagccggtgaacccgctggc	3891
Caprine AAV	..tC.a..gc.g.....t....C..C....g....	
Primate AAV type 5	gtacaacgtcggcgggcagatggccaccaacaaccagagctccaccactg	3941
Caprine AAV	t.....acg.....t.....gc....a.g.....g.	
Primate AAV type 5	cccccggaacgggcacgtacaacctccaggaatcgtgccccggcagcgtg	3991
Caprine AAV	.t...a..gt...g..C.....g.gc.t..t.....a	
Primate AAV type 5	tggatggagagggacgtgtacctccaaggacccatctgggccaagatccc	4041
Caprine AAV		
Primate AAV type 5	agagacggggcgcaactttcacccctctccggccatgggcggtatcgac	4091
Caprine AAV		
Primate AAV type 5	tcaaacacccacccgccatgatgtcatcaagsacacgcctgtgcccgga	4141
Caprine AAV	g.....a.....g.....C	
Primate AAV type 5	aatatcaccagcttctcggacgtgcccgtcagcagcttcatcaccagta	4191
Caprine AAV	..C.....	
Primate AAV type 5	cagcacccgggcaggtcacccgtggagatggagtgaggctcaagaaggaaa	4241
Caprine AAV	a.....a.....	
Primate AAV type 5	actccaagaggtggaacccagagatccagtacacaaacaactacaacgac	4291
Caprine AAV	C.....	
Primate AAV type 5	ccccagtttgtggactttgccccggacagcacccgggaatacagaaccac	4341
Caprine AAV	t..a...g..t...C.....	
Primate AAV type 5	cagacctatcggaacccgataccttaccgcacccctt	4378
Caprine AAV	g.C.....C.....	

FIGURE 12B

Primate AAV type 5 VP1	MSFVDHPPFDWLEEVEGGLREPLGLEAGFPKPKPNQOQBQQDQARGVLPGYNYLGPNGGLDR	60
Caprine AAV VP1		60
Primate AAV type 5 VP1	GEPVNRADAEVAEHDI SYNEQLEAGDNPYLYKNHADAEFQEKLA DDTSFSGNLGKAVPQA	120
Caprine AAV VP1		120
Primate AAV type 5 VP1	KRRVLEPFGLVEEGAKTAPTGRKRIDDHFFPKRKKA TEEDSKPSTSSDAEAGPSGSQQLQI	180
Caprine AAV VP1		180
Primate AAV type 5 VP1	PAQPASSILGADTMSAGGGGFLGDNNQGADGVGNASGDWHCDSTWMGDRVVTKTSTRTWLP	240
Caprine AAV VP1		240
Primate AAV type 5 VP1	SYNNHQYREIKSGSVDCSNANAYFGYSTPWGYPDFNRP SHSWSPRDWQRLINNYWGFRER	300
Caprine AAV VP1		300
Primate AAV type 5 VP1	SLRVKIFNIQVKEVTVDSTTTIANNLSTVQVTFDDDYQLFYVVGNGTEGCLFAFFPQV	360
Caprine AAV VP1		360
Primate AAV type 5 VP1	FTLPQGYATLNADNDENPTERSSFFCLEYFP SKMLATGNNFETTYNFEEVVFHSFAPS	420
Caprine AAV VP1		420
Primate AAV type 5 VP1	QNLPELANPLVDQYLRFVSTTGSGVQFNENLAGRIANTYKNWFP GPMGRTOGWNSG	480
Caprine AAV VP1	AI..Q.....	480
Primate AAV type 5 VP1	--GNRYSVPARTWRMLEGASYQVTPQPNGMTNKLQGSNYALENTMIFNSCANPGT	538
Caprine AAV VP1	SS...T...S... ..E...A...C...	540
Primate AAV type 5 VP1	TALYEINMLITSESETQPVNRVAYNPGGMATNLSSTAPACTYNLQEIVPGSVWME	598
Caprine AAV VP1	.SG...L.L... ..AA...VL...	600
Primate AAV type 5 VP1	RDVYLQGFPIWAKIPETGAHFHPSPAMGGFGLKHPPPMLIKNTVPVGNITSFSDVPVSSF	658
Caprine AAV VP1		660
Primate AAV type 5 VP1	ITQYSTGQVTVEMEWELKKENSERNPFIEIQYTNNYNDPQFVDFAPDRTGEYRTTPIGTR	718
Caprine AAV VP1		720
Primate AAV type 5 VP1	YLTRPL	724
Caprine AAV VP1		726

FIG. 13

	VP1	VP2
	50	
AAV-2	VP1:MAADGYLPDW LEDTLSEGIR QWWKLKPGPP PPKPAERHKD DSRGLVLPGY	
AAV-3B	VP1:MAADGYLPDW LEDNLSEGIR EWWALKPGVP QPKANQQHQD NRRGLVLPGY	
AAV-6	VP1:MAADGYLPDW LEDNLSEGIR EWWDLKPGAP KPKANQQKQD DGRGLVLPGY	
AAV-1	VP1:MAADGYLPDW LEDNLSEGIR EWWDLKPGAP KPKANQQKQD DGRGLVLPGY	
AAV-8	VP1:MAADGYLPDW LEDNLSEGIR EWWALKPGAP KPKANQQKQD DGRGLVLPGY	
AAV-4	VP1:M-TDGYLPDW LEDNLSEGVR EWWALQPGAP KPKANQQHQD NARGLVLPGY	
AAV-5	VP1:MSFVDHPPDW LEE-VGEGLR EFLGLEAGPP KPKPNQQHQD QARGLVLPGY	
Caprine AAV	VP1:MSFVDHPPDW LEE-VGEGLR EFLGLEAGPP KPKPNQQHQD QARGLVLPGY	
parvoviruses:*		
Other:		PPPPPPP
		100
AAV-2	VP1:KYLGPFGNGLD KGEVNEADA AALEHDKAYD RQLDSGDNFY LKYNHADAEEF	
AAV-3B	VP1:KYLGPFGNGLD KGEVNEADA AALEHDKAYD QQLKAGDNFY LKYNHADAEEF	
AAV-6	VP1:KYLGPFGNGLD KGEVNAADA AALEHDKAYD QQLKAGDNFY LRYNHADAEEF	
AAV-1	VP1:KYLGPFGNGLD KGEVNAADA AALEHDKAYD QQLKAGDNFY LRYNHADAEEF	
AAV-8	VP1:KYLGPFGNGLD KGEVNAADA AALEHDKAYD QQLQAGDNFY LRYNHADAEEF	
AAV-4	VP1:KYLGPFGNGLD KGEVNAADA AALEHDKAYD QQLKAGDNFY LKYNHADAEEF	
AAV-5	VP1:NYLGPFGNGLD RGEVNRAD E VAREHDISYN EQLEAGDNFY LKYNHADAEEF	
Caprine AAV	VP1:NYLGPFGNGLD RGEVNRAD E VAREHDISYN EQLEAGDNFY LKYNHADAEEF	
Parvoviruses:		*
Other:PPPPPPPPP	PPPPPPPPP	PPPPPPPPP

FIG. 14A

	VP1	VP2	
	150	13	
AAV-2	VP1:QERLKEDTSF	GGNLGRAVFQ	AKKRVLEPLG LVEEPVKTAP GKCRPVEHSP
AAV-3B	VP1:QERLQEDTSF	GGNLGRAVFQ	AKKRILEPLG LVEEAAKTAP GKCRPVDQSP
AAV-6	VP1:QERLQEDTSF	GGNLGRAVFQ	AKKRVLEPFG LVEEGAKTAP GKCRPVEQSP
AAV-1	VP1:QERLQEDTSF	GGNLGRAVFQ	AKKRVLEPLG LVEEGAKTAP GKCRPVEQSP
AAV-8	VP1:QERLQEDTSF	GGNLGRAVFQ	AKKRVLEPLG LVEEGAKTAP GKCRPVEPSP
AAV-4	VP1:QORLQGDTSF	GGNLGRAVFQ	AKKRVLEPLG LVEQAGETAP GKCRPLIESP
AAV-5	VP1:QEKLADDTSF	GGNLGKAVFQ	AKKRVLEPFG LVEEGAKTAP TGKRIDDHFP
Caprine AAV	VP1:QEKLADDTSF	GGNLGKAVFQ	AKKRVLEPFG LVEEGAKTAP TGKRIDDHFP
	Other:PPPPPPPPPP	PPPPPPPPPP	PPPP
AAV-2	VP1:VE-PDSSSGTG	KAGQQPARKR	LNFGQTGDAD SVDPDQPLGQ PPAAP-SGLGT 200 63
AAV-3B	VP1:QE-PDSSSGVG	KSGKQPARKR	LNFGQTGDSE SVDPDQPLGE PPAAP-TSLGS
AAV-6	VP1:QE-PDSSSGIG	KTGQQPAKKR	LNFGQTGDSE SVDPDQPLGE PPATP-AAVGP
AAV-1	VP1:QE-PDSSSGIG	KTGQQPAKKR	LNFGQTGDSE SVDPDQPLGE PPATP-AAVGP
AAV-8	VP1:QSPDSSSTGIG	KKGQQPARKR	LNFGQTGDSE SVDPDQPLGE PPAAP-SGVGP
AAV-4	VP1:QQ-PDSSSTGIG	KKGQQPAKKK	LVFEDETGA G DGPPEGSTSG AMSDD--S--
AAV-5	VP1:-----	KRKKARTEED	SKPSTSSDAE AGPSGSQQLO IPAQPASSLGA
Caprine AAV	VP1:-----	KRKKARTEED	SKPSTSSDAE AGPSGSQQLO IPAQPASSLGA

FIG. 14B

	VP1	VP2
/AAV-2 capsid structure begins here.		
AAV-2 VP1:NTMATGSGAP	MADNNEGADG VGNSSGNWHC DSTWMGDRVI	TTSTRTWALP 250 113
AAV-6 VP1:TTMASGGGAP	MADNNEGADG VGNASGNWHC DSTWLGDRVI	TTSTRTWALP
AAV-1 VP1:TTMASGGGAP	MADNNEGADG VGNASGNWHC DSTWLGDRVI	TTSTRTWALP
AAV-8 VP1:NTMAAGGGAP	MADNNEGADG VGNSSGNWHC DSTWLGDRVI	TTSTRTWALP
AAV-4 VP1:-EMRAAAGGA	AVEGGQADG VGNASGDWHC DSTWSEGHVT	TTSTRTWVLP
AAV-5 VP1:DTMSAGGGGP	LGDNNQADG VGNASGDWHC DSTWMGDRVV	TKSTRTWVLP
Caprine AAV VP1:DTMSAGGGGP	LGDNNQADG VGNASGDWHC DSTWMGDRVV	TKSTRTWVLP
Parvoviruses:	* *	*
Accessibility:	IIII IIIIIIIII IIIIIIIII IIIIBBBBBB	
DNA:	R P B	
Other:	M	
163		
AAV-2 VP1:TYNNHLYKQI	SSQS--GASNDN HYFGYSTPWG YFDENRFHCH	FSPRDWQRLI 300
AAV-3B VP1:TYNNHLYKQI	SSQS--GASNDN HYFGYSTPWG YFDENRFHCH	FSPRDWQRLI
AAV-6 VP1:TYNNHLYKQI	SSAST-GASNDN HYFGYSTPWG YFDENRFHCH	FSPRDWQRLI
AAV-1 VP1:TYNNHLYKQI	SSAST-GASNDN HYFGYSTPWG YFDENRFHCH	FSPRDWQRLI
AAV-8 VP1:TYNNHLYKQI	SNGTSGGATNDN TYFGYSTPWG YFDENRFHCH	FSPRDWQRLI
AAV-4 VP1:TYNNHLYKRL	GESL-----QSN TYNGFSTPWG YFDENRFHCH	FSPRDWQRLI
AAV-5 VP1:SYNNHQYREI	KSGSV-DGSNAN AYFGYSTPWG YFDENRFHSH	WSPRDWQRLI
Caprine AAV VP1:SYNNHQYREI	KSGSV-DGSNAN AYFGYSTPWG YFDENRFHSH	WSPRDWQRLI
Parvoviruses:	* *** * *	*
Neutralization:	# # # #	
Accessibility:OOOObO000	OBOO OOOOOO OOOBBBBBBB	BBBBBBBIBB
Surface Feature:YYY Y YY Y PP	PPPPPP PP	
Other:	AA	

FIG. 14C

	VP1	VP2
	350	213
AAV-2 VP1:NNWGFPRPKR	LNFKLFNIQV	KEVTQNDGTT TIANNLTSTV QVFTDSEYQL
AAV-3B VP1:NNWGFPRPKK	LSFKLFNIQV	KEVTQNDGTT TIANNLTSTV QVFTDSEYQL
AAV-6 VP1:NNWGFPRPKR	LNFKLFNIQV	KEVTINDGVT TIANNLTSTV QVFS DSEYQL
AAV-1 VP1:NNWGFPRPKR	LNFKLFNIQV	KEVTINDGVT TIANNLTSTV QVFS DSEYQL
AAV-8 VP1:NNWGFPRPKR	LSFKLFNIQV	KEVTQNEGK TIANNLTSTI QVFTDSEYQL
AAV-4 VP1:NNWGMRPKA	MRVKIFNIQV	KEVTSNGET TVANNLTSTV QIFADSSYEL
AAV-5 VP1:NNYWGFRPRS	LRVKIFNIQV	KEVTQDSTT TIANNLTSTV QVFTDDDYQL
Caprine AAV VP1:NNYWGFRPRS	LRVKIFNIQV	KEVTQDSTT TIANNLTSTV QVFTDDDYQL
Parvoviruses:	*	*
Accessibility: IIBIBBIIII	IIBIBBBBB	OOOOOOOOO OOBIBBBBB BBIIIIIIBB
Surface Feature:	CCCCCCCCC CC	
DNA: B D BB		PB D
Other:		M
AAV-2 VP1:PYVLGSAHQG	CLPPFPADVF	MVPQYGILTL N--NGSQ-AVGRS SFYCLEYFPPS 400
263		
AAV-3B VP1:PYVLGSAHQG	CLPPFPADVF	MVPQYGILTL N--NGSQ-AVGRS SFYCLEYFPPS
AAV-6 VP1:PYVLGSAHQG	CLPPFPADVF	MIPQYGILTL N--NGSQ-AVGRS SFYCLEYFPPS
AAV-1 VP1:PYVLGSAHQG	CLPPFPADVF	MIPQYGILTL N--NGSQ-AVGRS SFYCLEYFPPS
AAV-8 VP1:PYVLGSAHQG	CLPPFPADVF	MIPQYGILTL N--NGSQ-AVGRS SFYCLEYFPPS
AAV-4 VP1:PYVMDAGQEG	SLPPFPNDVF	MVPQGYCGL VTGNTSQQTDRN AFYCLEYFPPS
AAV-5 VP1:PYVVGNGTEG	CLPAFPQPQVF	TLPOYGYATL NRDN TEN-PTERS SFFCLEYFPPS
Caprine AAV VP1:PYVVGNGTEG	CLPAFPQPQVF	TLPOYGYATL NRDN GDN-PTERS SFFCLEYFPPS
Parvoviruses:	*	*
Accessibility: BBBBBBBBBB	BBBBBBBBBB	O OOOBBBBB BBBBBBBIII
Surface Feature:	Y YY P PPPP	
Other:	AA	

FIG. 14D

	VP1	VP2
AAV-2	VP1:QMLRTGNNFT	FSYTFEDVPP HSSYAHSQSL DRIMNPLIDQ YLYYLSRTN-T 450 313
AAV-3B	VP1:QMLRTGNNFQ	FSYTFEDVPP HSSYAHSQSL DRIMNPLIDQ YLYYLNRTQGT
AAV-6	VP1:QMLRTGNNFT	FSYTFEDVPP HSSYAHSQSL DRIMNPLIDQ YLYYLNRTQ-N
AAV-1	VP1:QMLRTGNNFT	FSYTFEEVPP HSSYAHSQSL DRIMNPLIDQ YLYYLNRTQ-N
AAV-8	VP1:QMLRTGNNFQ	FTYTFEDVPP HSSYAHSQSL DRIMNPLIDQ YLYYLSRTQ-T
AAV-4	VP1:QMLRTGNNFE	ITYSFEKVPP HSMYAHSQSL DRIMNPLIDQ YLWGLQSTT-T
AAV-5	VP1:KMLRTGNNFE	FTYNFEEVPP HSSFAPSQNL FKLANPLVDQ YLYRFVSTN-N
Caprine AAV	VP1:KMLRTGNNFE	FTYSFEEVPP HSSFAPSQNL FKLANPLVDQ YLYRFVSTS-A
Parvoviruses: *		
Accessibility:	IIBBBIII	IIBBBIBOB OBEBBBOOB BBBBBBOBB O
Surface Feature:		P P P YYY S S
DNA:	P B BB D D D	
AAV-2	VP1:PSGTTTQSRL	QFSQAGASDI RDQSRNWLPG PCYRQQRVSK TSA-----DNNNSEY 500
AAV-3B	VP1:TSGTTNQSRL	LFSQAGPQSM SLQARNWLPG PCYRQQRVSK TAN-----DNNNSNF
AAV-6	VP1:QSGSAQNKDL	LFSRGSPAGM SVQPKNWLPG PCYRQQRVSK TKT-----DNNNSNF
AAV-1	VP1:QSGSAQNKDL	LFSRGSPAGM SVQPKNWLPG PCYRQQRVSK TKT-----DNNNSNF
AAV-8	VP1:TGGTANTQTL	GFSQGGPNTM ANQAKNWLPG PCYRQQRVST TTG-----QNNNSNF
AAV-4	VP1:TTLNAGTATT	NFTKLRPNTF SNFKKNWLPG PSIKQQGFSK TANQNYKIPATGSDS
AAV-5	VP1:TGG-----V	QFNKNLAGRY ANTYKNWFPG PMGRTQGNL GSG-----VNRASVS
Caprine AAV	VP1:TGA-----I	QFQKNLAGRY ANTYKNWFPG PMGRTQGNNT SSGSSTNRVSVN
Neutralization: #		# # #
Accessibility: OOOOOOOO	OBEBBBOOB	BBOBBOOB OOO OBOOOB
Surface Feature: SSSSSSSS	S PPPP PP	SS SS S SSS S SSSS
Other:		H H

FIG. 14E

	VP1	VP2
	550	413
AAV-2	VP1:SWTGATKYHL NGRDSLVPNG PAMASHKDDE EKFPQSGVL IFGKQSEKT	
AAV-3B	VP1:PWTAASKYHL NGRDSLVPNG PAMASHKDDE EKFPFPMHGNL IFGKEGTAS	
AAV-6	VP1:TWIGASKYNL NGRESIINPG TAMASHKDDK DKFFPMSGVM IFGKESAGAS	
AAV-1	VP1:TWIGASKYNL NGRESIINPG TAMASHKDDDE DKFFPMSGVM IFGKESAGAS	
AAV-8	VP1:AWTAGTKYHL NGRNSLANPG IAMATHKDDDE ERFFPSNGIL IFGKQNAARD	
AAV-4	VP1:LIKYETHSTL DGRWSALTGP PPMATAGPAD SKFSNSQLIF AGPKQNGNTA	
AAV-5	VP1:AFATTNRMEL EGASYQVPPQ PNGMTNNLQG SNTYALENTM IFNSQPANPG	
Caprine AAV	VP1:NFSVSNRMNL EGASYQVNPQ PNGMTNTLQG SNRYALENTM IFNAQNATPG	
Neutralization:	# # # # #	# # #
Accessibility:	BOOBBOBOB OOBBOBBOB BBBBBBOOOO OOBBOBBOB BBBBBBOOOO	BBBOOOBOOO
Surface Feature:	PP P P PP P	PPPP PSS SSS SSS
Other:		HH A
AAV-2	VP1:NVDI--E-KVMIT DEEEIRTTNP VATEQYGSVS TNLQGNRQA ATADVNTQGV	600
463		
AAV-3B	VP1:NAEL---DNVMIT DEEEIRTTNP VATEQYGTVA NNQSSNTAP TTRTVNDQGA	
AAV-6	VP1:NTAL---DNVMIT DEEEIKATNP VATERFGTVA VNLQSSSTDP ATGDVHMGA	
AAV-1	VP1:NTAL---DNVMIT DEEEIKATNP VATERFGTVA VNFQSSSTDP ATGDVHMGA	
AAV-8	VP1:NADY---SDVMLT SEEEIKTTNP VATEEYGTVA DNLOQQNTAP QIGTVNSQGA	
AAV-4	VP1:TVPG---TLIFT SEEEELAAATNA TDTDMWGNLP GGDQSNNSLP TVDRRLTALGA	
AAV-5	VP1:TTATYLEGNMLIT SESETQPVNR VAYNVGGQMA TNNQSSSTAP ATGTYNLQEI	
Caprine AAV	VP1:TTSVYPEDNLLIT SESETQPVNR VAYNTGGQMA TNAQNATTAP TVGTYNLQEV	
Neutralization:	#	##
Accessibility:	OOOO O OBOB OBOBOBBOB BBOBOBBOB OOBBOBOBOB OOBBOBOBOB	OOOBBOBOBB
Surface Feature:	SSSS S SS S Y YY Y	PP P SSS SSSS PPPP PPP
Other:		H H H

FIG. 14F

	VP1	VP2
	650	513
AAV-2 VP1:LPGMVWQDRD	VYLQGPWAK	IPHTDGHFHP
AAV-3B VP1:LPGMVWQDRD	VYLQGPWAK	IPHTDGHFHP
AAV-6 VP1:LPGMVWQDRD	VYLQGPWAK	IPHTDGHFHP
AAV-1 VP1:LPGMVWQDRD	VYLQGPWAK	IPHTDGHFHP
AAV-8 VP1:LPGMVWQNRD	VYLQGPWAK	IPHTDGNFHP
AAV-4 VP1:VPGMVWQNRD	IYYQGPWAK	IPHTDGHFHP
AAV-5 VP1:VPGSVWMERD	VYLQGPWAK	IPETGAHFHP
Caprine AAV VP1:LPGSVWMERD	VYLQGPWAK	IPETGAHFHP
Parvoviruses:	* *	* *
Accessibility:BBBBBIBBO	BBBBBBIBBI	IBIIIIIIII
Surface Feature:	S	
DNA:	P	B
		BB B
AAV-2 VP1:TPVPANPSTT	FSAAKFASFI	TOYSTGQVSV
AAV-3B VP1:TPVPANPPTT	FSPAKFASFI	TOYSTGQVSV
AAV-6 VP1:TPVPANPPAE	FSATKFASFI	TOYSTGQVSV
AAV-1 VP1:TPVPANPPAE	FSATKFASFI	TOYSTGQVSV
AAV-8 VP1:TPVPANPSTT	FSAAKFASFI	TOYSTGQVSV
AAV-4 VP1:TPVPANPATT	FSSTPVNSFI	TOYSTGQVSV
AAV-5 VP1:TPVPGN-ITS	FSDVPVSSFI	TOYSTGQVTV
Caprine AAV VP1:TPVPGN-ITS	FSDVPVSSFI	TOYSTGQVTV
Parvoviruses:	*	*
Accessibility:BBBBBOBBOO	OBOOOOOOOO	OBOBBBBBII
Surface Feature:	YY YY YY YYYYYY YY	YY
DNA:		P
		BB B
AAV-2 VP1:TPVPANPSTT	FSAAKFASFI	TOYSTGQVSV
AAV-3B VP1:TPVPANPPTT	FSPAKFASFI	TOYSTGQVSV
AAV-6 VP1:TPVPANPPAE	FSATKFASFI	TOYSTGQVSV
AAV-1 VP1:TPVPANPPAE	FSATKFASFI	TOYSTGQVSV
AAV-8 VP1:TPVPANPSTT	FSAAKFASFI	TOYSTGQVSV
AAV-4 VP1:TPVPANPATT	FSSTPVNSFI	TOYSTGQVSV
AAV-5 VP1:TPVPGN-ITS	FSDVPVSSFI	TOYSTGQVTV
Caprine AAV VP1:TPVPGN-ITS	FSDVPVSSFI	TOYSTGQVTV
Parvoviruses:	*	*
Accessibility:BBBBBOBBOO	OBOOOOOOOO	OBOBBBBBII
Surface Feature:	YY YY YY YYYYYY YY	YY
DNA:		P
		BB B
AAV-2 VP1:TPVPANPSTT	FSAAKFASFI	TOYSTGQVSV
AAV-3B VP1:TPVPANPPTT	FSPAKFASFI	TOYSTGQVSV
AAV-6 VP1:TPVPANPPAE	FSATKFASFI	TOYSTGQVSV
AAV-1 VP1:TPVPANPPAE	FSATKFASFI	TOYSTGQVSV
AAV-8 VP1:TPVPANPSTT	FSAAKFASFI	TOYSTGQVSV
AAV-4 VP1:TPVPANPATT	FSSTPVNSFI	TOYSTGQVSV
AAV-5 VP1:TPVPGN-ITS	FSDVPVSSFI	TOYSTGQVTV
Caprine AAV VP1:TPVPGN-ITS	FSDVPVSSFI	TOYSTGQVTV
Parvoviruses:	*	*
Accessibility:BBBBBOBBOO	OBOOOOOOOO	OBOBBBBBII
Surface Feature:	YY YY YY YYYYYY YY	YY
DNA:		P
		BB B

FIG. 14G

		<u>VP1</u>	<u>VP2</u>
		735	598
AAV-2 capsid structure ends here \			
AAV-2	VP1:TSNYNKS	VNV	DFTVD
AAV-3B	VP1:TSNYNKS	VNV	DFTVD
AAV-6	VP1:TSNYAKS	ANV	DFTVD
AAV-1	VP1:TSNYAKS	ANV	DFTVD
AAV-8	VP1:TSNYK	STSV	DFAVN
AAV-4	VP1:TSNYG	QONSL	LWAPD
AAV-5	VP1:TNNDP	QFV	DFAPD
Caprine AAV	VP1:TNNDP	QFV	DFAPD
Neutralization:	##	##	
Accessibility:	O	O	O
Surface Feature:	Y	Y	Y
DNA:			
DNA:			
Other:			

FIG. 14H

```

      "creek"
            OO          D      I
Primate AAV-5 FTLPGQYGYATLNARDNTENPTERSSTFCLEYFPFSKMLRTGNNFTFTYNFEEVPPHSSSFAPS    420
Caprine AAV .....[X].[X].....[X].[X].                                420

               "SPIKE" (7 aa deletion vs. AAV2) "SPIKE"
                     <---->                      <----->
                   II OI O                          H H OO
QNLFKLANPLVDQYLRFVSTNNTGGVQFNKNLAGRYANTYENWFGPMGRTOGWNLSSG    480
.....[X].[X].[X].....[X]..                                480


           "SPIKE"                    "PLATEAU"                    "SPIKE"
       <----->             <----->             <----->
                                     (3 aa ins)
OOO O II OII O         D         O HO        O OOO
--VNRRASVSFAFATTNRMELEGASYQVPQPNGMTNNLQGSNTYALENTMIFNSQPANFGT    538
[XX].[X].[X].[X].[X].[X].[X].[X].[X].[X].[X].[X].[X].[X].[X].[X].[X]...[X].[X].[X]... 540


           "SPIKE"                    "SPIKE"
       <----->             <----->
                               H H H
OO O O O O              O          O OO      OO      IX

```

FIG. 15

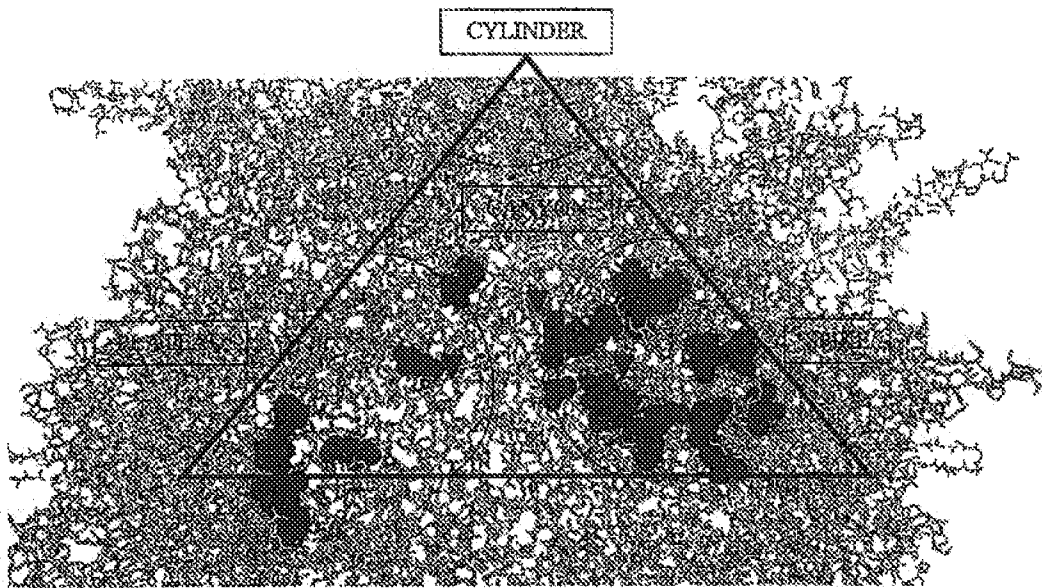


FIG. 16

FIGURE 17

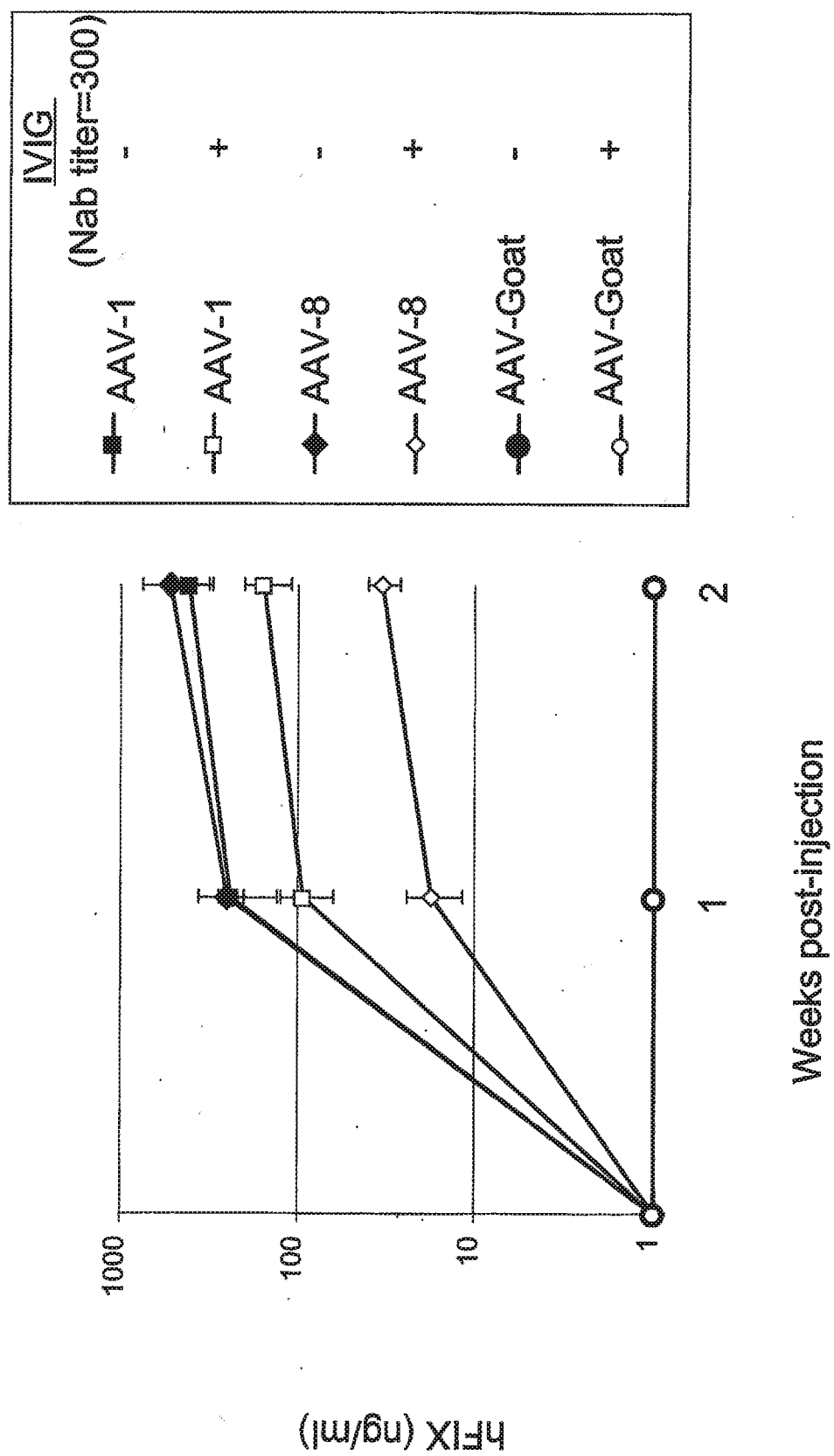


FIGURE 18

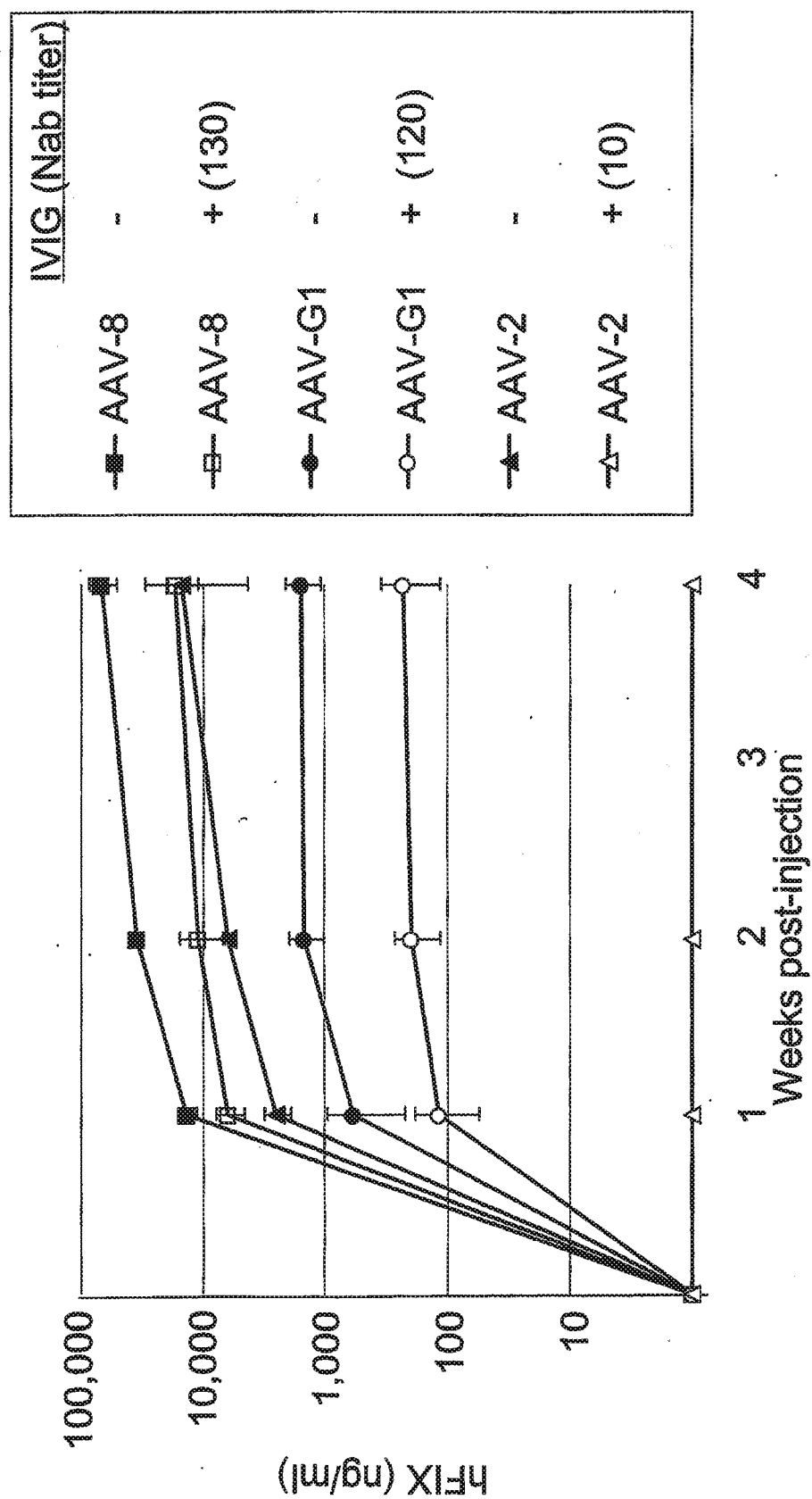
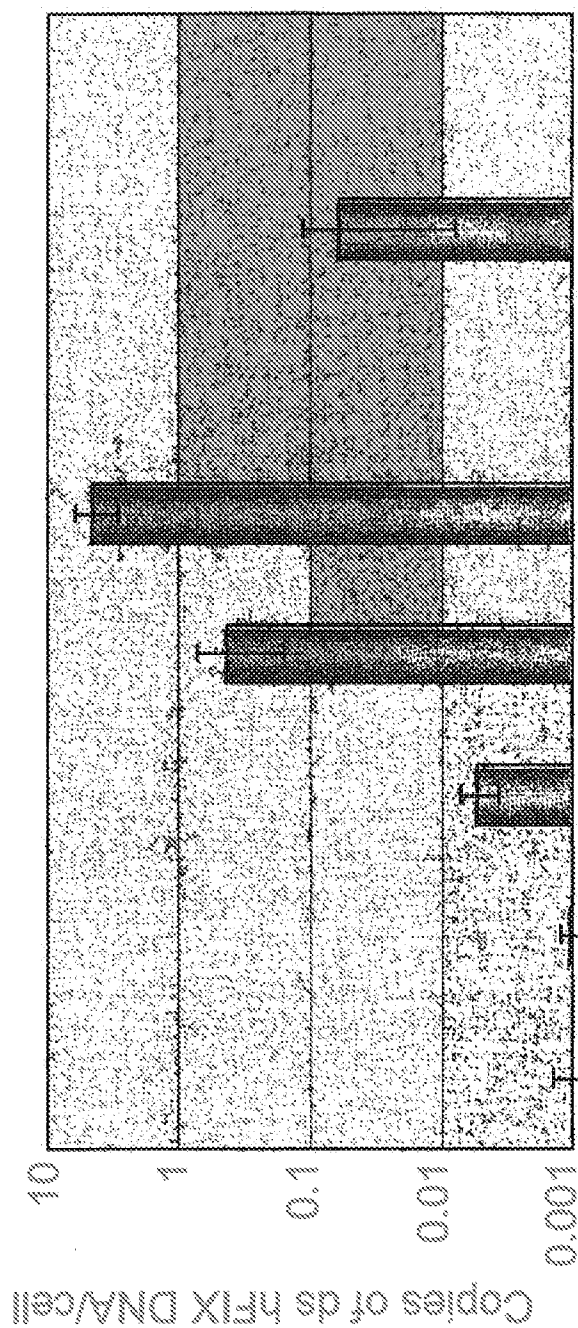


FIGURE 19



	Brain	Heart	Kidney	Liver	Lung	Muscle	Spleen	Testis
AAV-G1 hFIX (n=2)	0.0008	0.001	0.005	0.43	4.52	< 0.0005	0.060	0.0005

ATGTCTTTTGTGACCACTCCAGATTGGTTGGAATCGATCGCGGACGGCTTTCGTGAATTTCTCGGCCTTGAGGCGGG 80
 TCCCCCGAAACCAAGGCCAATCAACAGAGCAAGATACCGCTCGAGGCTTTGTGCTTCTGGGTACAAGTATCTTGGTC 160
 CTGGGAACGGCCTTGATAAGGGCGATCCTGTCAATTTTGTGACGAGGTTGCCCCGAGAGCACGACCTCTCCTACCAGAAA 240
 CAGCTTGAGGCGGGCGATAACCTTACCTCAAGTACAAACCGCGGACCGCAGAGTTTCAGGAGAAATCTCGCTTCTGACAC 320
 TTCTTTTGGAGGAAACCTTGGGAAGGCTGTTTTCAGGCTAAAAAGAGGATTTCTCGAACCTCTTGGCTTGGTTGAGACGC 400
 CGGATAAAACCGCGCTGCGGCAAAAAGAGGCTCTAGAGCAGAGTCTCAAGAGCCAGACTCCTCGAGCGGAGTTGGC 480
 AAGAAAGGCAACAGCCTGCCAGAAAGAGACTCAACTTTGACGACGAACTGGAGCGGAGACGGGCTCCCCAGAAAGG 560
 ACCATCTTCCGGAGCTATGTCTACTGAGACTGAAATGCGTGCAGCAGCTGGCGGAAATGGTGGCGATGCGGACAAAGGTG 640
 CCGAGGGAGTGGGTAATGCCTCCGGTGATTGGCATTGCGATTCCACTTGGTCAGAGAGCCACGTCACCACCACCTCAACC 720
 CGCACCTTGGGTCTTGGCGACCTACAAACAACCACTGTACCTTGGCGCTCGGCTCGAGCAACGCCAGCGACACCTTCAACGG 800
 ATTCTCCACCCCTGGGGATACTTTGACTTTAACCCTTCCACTGCCACTTCTGCCAAGAGACTGGCAAAGGCTCATCA 880
 ACAACCACTGGGGACTGCGSCCAAAAGCATGCAAGTCCGCATCTTCAACATCCAGTTAAGGAGGTCACGACGTCTAAC 960
 GGGGAGACGACCGTATCCAAACAACCTCACCAGCACGCTCCATATCTTTGCGGACAGCAGTACGAGCTCCCGTACGTGAT 1040
 GGATGCAAGTTCAGGAGGGCAGCTTGCTCTCTTCCCCAACGACGTGTTTCATGGTGCCTCAGTACGGGTACTGCGGACTGG 1120
 TAACCGGAGGCGAGCTCTCAAAACAGACAGACAGAAATGCTTCTACTGTCTGGAGTACTTTCCAGCCAGATGCTGAGA 1200
 ACCGGAACAACCTTTGAGATGGTGTACAAGTTTGAACAGTGGCCTTCCACTCCATGTACGCTCACAGCCAGAGCCTGGA 1280
 TAGGCTGATGAACCCGCTGCTGGACCACTGCTGGGAACCTCCAGTCTACCACTCTGGAGGAACCTCAACCCAGGGCA 1360
 ATTCAGCCACCACTTTGCCAAGCTGACCAACAAAACCTTTCTGGTACCGCAAAAACCTGGCTCCCGGGGCCCATGATG 1440
 AAGCAGCAGAGATTCTCAAGACTGCCAGTCAAACTACAAGATTCCCCAGGAGGAAACACAGTCTGCTCATTTATGA 1520
 GACCAGAACTACCTCGACAGAAATGGAGCAATTTGCCCCGGGAACGGCCATGCCAACCAGCCAGCCAGACGCCACCG 1600
 ACTTCTCAGGCGCCAGCTCATCTTTGCGGGGCCCAACATCACCGGCAACACCAACACAGATGCCAATAATCTGATGTTT 1680
 ACTTCAGAAATGAACCTTAGGGCCACCAACCCCCGGGACACTGACCTGTTTGGCCACCTGGCAACCAACAGCAAAACGC 1760
 CACCACCGTTCTACCGTAGACGAGCTGGACGGAGTCCGGCTGTACCGGGAATGGTGTGGCAGGACAGACATTTACT 1840
 ACCAAGGGCCCAATTTGGGCCAAAATTCACACACGAGATGGACACTTTCACCCGCTCTCTCTCATTTGGCGGATTGGACTG 1920
 AAAAGCCCGCCTCCACAAATATTCATCAAAACACTCCTGTACCCGCCAATCCCGCAACGACCTTCTCTCCGGCCAGAAT 2000
 CAACAGCTTCATACCCAGTACAGCACCGGACAGGTGGCTGTCAAAATAGAAATGGGAATCCAGAAAGAGCGGTCCAAGA 2080
 GATGGAACCCAGAGGCTCAGTTCACGTCCAACCTACGGAGCACAGGACTCGCTTCTCTGGGCTCCCGCAACGCCGAGCC 2160
 TACAAAGAGCCCGAGGCCATTGGATCCCGATACCTCACAACCACTCTAG 2211

FIG 20A

MSFVDHPPDWLESIGDGFREFLGLLEAGPPKPKANQQKQDNARGLVLPYK 50
 YLPGNGLDKGDVNFADDEVAREHDLISYQKQLEAGDNPYLKYNHADAEFQ 100
 EKLASDTSFGGNLGAVFQAKKRILEPLGLVETPDKTAPAAKKRPLEQSP 150
 QEPDSSSGVGKKGKQPARKRLNFDDEPGAGDGPPEGPSSGAMSTETEMR 200
 AAAGNGGDAGQGAEGVGNASGDWHCDSTWSESHVTTTSTRTWVLPYNN 250
 HLYLRLGSSNASDTFNGFSTPWGYFDNRFHCHFSPRDWQRLINNHWGLR 300
 PKSMQVRIFNIQVKEVTTSNGETTVSNNLTSTVHIFADSTYELPYVMDAG 350
 QEGSLPPFPNDVFMVPQYGYCGLVTGGSSQNQTDNFAFYCLEYFPSQMLR 400
 TGNNFEMVYKFENVPFHSMYAHQSLSLDRMLNPLLDQYLWELQSTTSGGTL 450
 NQGN SATNFAKL TNKNFSGYRKNWLP GPM MKQRF SKTASQNYKI PQGN 500
 NSLLHYETRTTLD RRSNFAPGTAMATAANDATDFSQAQLIFAGPNITGN 550
 TTTDANNLMFTSEDEL RATNPRDTDLFGHLATNQNNATTVP TVDDVDVG 600
 VYPGMVWQDRDIYYQGP IWAKI PHTDGHFHFSPLIGGFGLKSPPPQIFIK 650
 NTPVPANPATTFSPARINSFITQYSTGQVAVKIEWEIQKERSKRWNPEVQ 700
 FTSNYGAQDSLLWAPDNAGAYKEPRAIGSRYLTNHL 736

FIG 20B

	VP1	VP2
	50	
AAV-2 VP1:MAADGYLPDW LEDTLSEGIR QWNKPKPGPP PPKPAERHKD DSRGLVLPGY		
AAV-3B VP1:.....N.....E..A.....Q..A.....		
AAV-6 VP1:.....N.....E..D....A..K..ANQKQ..G.....		
AAV-1 VP1:.....N.....E..D....A..K..ANQKQ..G.....		
AAV-8 VP1:.....N.....E..A....A..K..ANQKQ..G.....		
AAV-4 VP1:..-T.....V..E..A.Q..A..K..ANQKQ..NA.....		
AAV-C1 VP1:..SFVDHP...S-IGD.F..EFLG.EA...K..KANQKQ..NA.....		
AAV-5 VP1:..SFVDHP...E-VG..L..EFLG.EA...K...NQK.Q..QA.....		
AAV-G1 VP1:..SFVDHP...E-VG..L..EFLG.EA...K...NQK.Q..QA.....		
Parvoviruses:*		
Other:		PPPPPPP
AAV-2 VP1:KYLGPFNGLD KGEVPNEADA AALEHDKAYD RQLDSGDNPY LKYNHADAEEF 100		
AAV-3B VP1:.....G....		Q..KA.....
AAV-6 VP1:.....A....		Q..KA.....
AAV-1 VP1:.....A....		Q..KA.....
AAV-8 VP1:.....A....		Q..QA.....
AAV-4 VP1:.....G....		Q..KA.....
AAV-C1 VP1:.....G....D...F..E..V.R...LS.Q..K..EA.....		
AAV-5 VP1:N....G....R....R..E..V.R...IS.N..E..EA.....		
AAV-G1 VP1:N....G....R....R..E..V.R...IS.N..E..EA.....		
Parvoviruses:	*	** *
Other:PPPPPPPPPP	PPPPPPPPPP	PPPPPPPPPP

FIG. 21A

	VP1	VP2	VP1	VP2
AAV-2	VP1:QERLKEDTSF	GGNLGRAVFQ	AKKRVLEPLG	LVEEVPKTAP G-KKRPVEHSP 150 13
AAV-3B	VP1:....Q		I....	AA....-....DQ..
AAV-6	VP1:....Q		F	GA....-....Q..
AAV-1	VP1:....Q			GA....-....Q..
AAV-8	VP1:....Q			GA....-....P..
AAV-4	VP1:..Q..QG			QAGE....-....IE..
AAV-C1	VP1:..K.AS	K....	I....	T.D....AA....L.Q..
AAV-5	VP1:..K.AD	K....	F	GA....T-G..IDD.F.
AAV-G1	VP1:..K.AD	K....	F	GA....T-G..IDD.F.
Other:PPPPPPPPPP PPPPPPPPP PPPPPPPPP PPPPP				
AAV-2	VP1:VE-PDSSSGTG	KACQOPARKR	INFGQTGDAD	SVPD-PQPLGQ PPAAP-SGLGT 200 63
AAV-3B	VP1:Q-....V	S.K....	SE	-....E....-TS..S
AAV-6	VP1:Q-....I	T....K..	SE	-....E....T-AAV.P
AAV-1	VP1:Q-....I	T....KKR	SE	-....E....T-AAV.P
AAV-8	VP1:QRS	T.I.	K....	SE....-....V.P
AAV-4	VP1:QQ-....T.I	K.K...K.K	V.EDETG.G	DG.P-EGSTSG AMSDD-....
AAV-C1	VP1:Q-....V	K.K....	...DDEPG.G	DG.P-EGPSSG AMSTE-T-....
AAV-5	VP1:-----	RKKARTEED	SKPSTSS..E	AGPS-GSQQL I..Q.A.S..A
AAV-G1	VP1:-----	RKKARTEED	SKPSTSS..E	AGPS-GSQQL I..Q.A.S..A

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	VP1	VP2
/AAV-2 capsid structure begins here.		
AAV-2 VP1:NTMATGSGAP	MADNNEGADG VGNSSGNWHC	DSTWMGDRVI TTSTRTWALP 250 113
AAV-3B VP1:.....S.G...	Q.L.....	
AAV-6 VP1:T...S.G...	A.....L.....	
AAV-1 VP1:T...S.G...	A.....L.....	
AAV-8 VP1:.....A.G...	SS.....L.....	
AAV-4 VP1:-E.RAAA.GA AVEGGQ....	A.D....SEGH.T	V..
AAV-C1 VP1:-E.RAAA.GN GG.AGQ..E.	A.D....SESH.T	V..
AAV-5 VP1:D..SA.G.G. LG...Q....	A.D....K.....V..	
AAV-G1 VP1:D..SA.G.G. LG...Q....	A.D....K.....V..	
Parvoviruses: *	*	*
Accessibility:	IIII IIIIIIIII IIIIIIIII IIIIBBBBBB	
DNA:	R P B	
Other:	M	
AAV-2 VP1:TNNHLYKQI	SSQS--GASNDN HYFGYSTPWG	YFDNRFHCH FSPRDWQRLI 300 163
AAV-3B VP1:.....A.T-		
AAV-6 VP1:.....A.T-		
AAV-1 VP1:.....NGTSG..T...	T.....	
AAV-8 VP1:.....RL GESL-----QS.	T.N.F.....	
AAV-4 VP1:.....LRL G.SN-----ASD TFN.F		
AAV-C1 VP1:S...Q.RE. K.G.V-DG..A.	S. W.....	
AAV-G1 VP1:S...Q.RE. K.G.V-DG..A.	S. W.....	
Parvoviruses: *	***	* * *
Neutralization:	# # # #	
Accessibility:OOOOBOBOOO OBOO	OOOOOO OOBEBEBEBB	EBEBEBEBB BBBBBBBIBB
Surface Feature:YYY Y YY Y PP	PPPPPP PP	
Other:	AA	

FIG. 21C

	VP1	VP2
	350	213
AAV-2 VP1:NNNWGERPKR LNEKLENIQV KEVTONDGTT TIANNLTSTV QVFTDSEYQL		
AAV-3B VP1:.....K .S.....		
AAV-6 VP1:.....T...V.....		
AAV-1 VP1:.....T...V.....		
AAV-8 VP1:.....S.....E..K.....I.....		
AAV-4 VP1:.....M...A MRV.I.....TSN.E. V.....I.A..S.E.		
AAV-C1 VP1:..H..L...S MQVRI.....TSN.E. .VS.....HI.A..T.E.		
AAV-5 VP1:..Y.....RS LRV.I.....VQ.S.....DD...		
AAV-G1 VP1:..Y.....RS LRV.I.....VQ.S.....DD...		
Parvoviruses:	*	*
Accessibility:IIIBIBIIII IIIBIBBBBB OOOOOOOOOO OOBIBBBBB BBIIIIIIBB		
Surface Feature:	CCCCCCCCC CC	
DNA: B D BB		PB D
Other:		M

	VP1	VP2
	350	213
AAV-2 VP1:PYVLGSAHQG CLPPEPADVF MVPQGYLTL N--NGSQ-AVGRS SFYCLEYFPS		400/263
AAV-3B VP1:.....I.....		
AAV-6 VP1:.....I.....		
AAV-1 VP1:.....I.....		
AAV-8 VP1:.....I.....		
AAV-4 VP1:..MDAGQE. S.....N...CG. VTG.T..QQTD.N A.....		
AAV-C1 VP1:..MDAGQE. S.....N...CG. VTG..S.NQTD.N A.....		
.AAV-5 VP1:..V.NGTE. ...A..PQ.. TL.....A...RD.TEN-PTE.. .F.....		
AAV-G1 VP1:..V.NGTE. ...A..PQ.. TL.....A...RD..DN-PTE.. .F.....		
Parvoviruses:	*	*
Accessibility:BIIBBBBBBB BBBBBBBBBB OOOBBBBBB BBBBBBBIIII		
Surface Feature:	Y YY P PPPP	
Other:	AA	

FIG. 21D

STIG.

VP1 VP2
550 413

AAV-2 VP1:SWTGATKYHL NGRDSLVPNG PAMASHKDDE EKFFPQSGVL IFGKQSGSEKT
AAV-3B VP1:P..A.S.... MH.N. E.TTAS
AAV-6 VP1:T...S.N. ...E.II... T.....K D....M...M ESAGAS
AAV-1 VP1:T...S.N. ...E.II... T.....D....M...M ESAGAS
AAV-8 VP1:A..AG..... I...T..... R...SN.I. NAARD
AAV-4 VP1:LIKYE.HST. D..W.ALT.. PP..TAGPAD S..SNSQLIF AGP..NGNTA
AAV-C1 VP1:LLHYE.RTT. DR.W.NFA.. T...TAAN.A TD.SQAQLIF AGENITGNT.
AAV-5 VP1:AFATTNRME. E.ASYQ.P.Q PNGMTNNLQG SNTYALENTM ..NS.PANPG
AAV-G1 VP1:NFSVSNRMN. E.ASYQ...Q PNGMTNTLQG SNRYALENTM ..NA.NATPG
Neutralization: # # # # # # # #
Accessibility:BOOBBOBOB OOBBOBOBOO OOBBOBOBOO BBBOBOBOBO BBBOBOBOBO
Surface Feature: PP P P PP P PPPP PSS SSS SSS
Other: HH A

AAV-2 VP1:NVDI--E-KVMIT DEEEIRTTNP VATEQYGSVS TNLQRGNROA ATADVNTQGV 600463
AAV-3B VP1:.AEL---DN.... KA.... RF.T.A V...SSSTD P A.G..HVM.A
AAV-6 VP1:.TAL---DN.... KA.... RF.T.A V...SSSTD P A.G..HVM.A
AAV-1 VP1:.TAL---DN.... KA.... RF.T.A V...SSSTD P A.G..HVM.A
AAV-8 VP1:.A.Y---SD..L. S....K.... E..I.A D...QQ.TAP QIGT..S..A
AAV-4 VP1:T.PG---TLIF. S...LAA..A TD.DMW.NLP GGD.SNSNLP TVDRLLTAL.A
AAV-C1 VP1:TDAN---NL.F. S.D.L.A... RD.DLE.HLA ..Q.NATTVP TVD..DGV..
AAV-5 VP1:TTATYL.GNML.. S.S.TQPV.R ..YNVG.QMA ..N.SSTTAP ..GTY.L.EI
AAV-G1 VP1:TTSVYP.DNLLL. S.S.TQPV.R ..YNTG.QMA ..A.NATTAP TVGTY.L.E.
Neutralization: # # #
Accessibility:OOOO O OOB OBOBOBOBB BBBOBOBOBB OOBBOBOBOBB OOBBOBOBOBB
Surface Feature:SSSS S SS S Y YY Y PP P SSS SSSSS PPPP PPP
Other: H H H

FIG. 21F

	VP1	VP2
	650	513
AAV-2 VP1: LPCMVWQDRD VYLQGPWAK IPHTDGHHP SPLMGFGCLK HPPQILIKN		
AAV-3B VP1:		M...
AAV-6 VP1:		
AAV-1 VP1:		N
AAV-8 VP1:	N	
AAV-4 VP1: V.....	I.Y.....	I.....F...
AAV-C1 VP1: Y.....	I.Y.....	I.....S.....F...
AAV-5 VP1: V..S..ME..	..E.GA....	..A.....MM....
AAV-G1 VP1: L..S..ME..	..E.GA....	..A.....MM....
Parvoviruses: *	* *	* *
Accessibility: BBBBBIIBBO BBBBBIIBBI IBIIIIIIII IBBBBBBBII IIIBBBBBBB		
Surface Feature: S		
DNA:	P	BB B
AAV-2 VP1: TEVPANPSTT ESAAKFASFI TQYSTGQSV EIEWELOKEN SKRWNPFIQY		700 563
AAV-3B VP1:	P.....	
AAV-6 VP1:	PAE	V...
AAV-1 VP1:	PAE	V...
AAV-8 VP1:	..STPVN...	Q.D..I....V.F
AAV-4 VP1:	..P.RIN...	A.K..I....V.F
AAV-C1 VP1:	..G.-I.S..DVPVS...	M....K....
AAV-5 VP1:	..GN-I.S..DVPVS...	M....K....
AAV-G1 VP1:	..DVPVS...	M....K....
Parvoviruses:	*	* *
Accessibility: BBBBBOBBOO OOBBOBBOO OOBBOBBOO OOBBOBBOO		IIIIIIIIII IIIBBBBBOO
Surface Feature: YY YY YY YY YY YY YY YY		YYY P
DNA:		

FIG. 21G

	VP1	VP2
AAV-2 VP1:TSNYNKSVNV	735	598
AAV-3B VP1:.....		
AAV-6 VP1: ...A.A..		
AAV-1 VP1: ...A.A..		
AAV-8 VP1: ...Y.TS.		
AAV-4 VP1: ...GQONSL		
AAV-C1 VP1: ...GAQDSL		
AAV-5 VP1: ...N...DPQF.		
AAV-G1 VP1: ...N...DPQF.		
Neutralization: ## #		
Accessibility:OB0000000B		
Surface Feature:Y YYYPYP Y		
DNA:		
DNA:		
Other: A		

FIG. 12

REFERENCES CITED IN THE DESCRIPTION

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Szabadalmi igénypontok

1. Rekombináns adeno-asszociált vírus (AAV) virionban lévő mutált AAV kapszid protein alkalmazása a virionnak a megfelelő vad típusú virionhoz hasonlított immunreaktivitásának csökkentésére, ahol a mutált AAV kapszid protein azon aminosav szubsztitúcióját tartalmazza, amely az AAV-2 VP2 kapszid proteinje 334. pozíciójának felel meg.
2. Az 1. igénypont szerinti alkalmazás, ahol az AAV-2 VP2 kapszid proteinje 334. pozíciójának megfelelő pozícióban előforduló aminosav alaninnal van szubsztituálva.
3. Az 1. vagy 2. igénypont szerinti alkalmazás, ahol a rekombináns AAV virion heterológ nukleinsav-molekulát tartalmaz.
4. Az 1-3. igénypontok bármelyike szerinti alkalmazás, ahol a rekombináns AAV virion heterológ nukleinsav-molekulát tartalmaz, amely antiszensz RNS-t vagy ribozimot kódol.
5. Az 1-4. igénypontok bármelyike szerinti alkalmazás, ahol a rekombináns AAV virion heterológ nukleinsav-molekulát tartalmaz, amely terápiás proteint kódol, működőképesen kapcsoltan olyan szabályozó elemekhez, amelyek képesek irányítani a terápiás protein *in vivo* transzkripcióját és transzlációját.
6. Az 5. igénypontban definiált csökkentett immunreaktivitású rekombináns adeno-asszociált vírus (AAV) virion, gerinces alany kezelési eljárásában történő alkalmazásra, ahol a heterológ nukleinsav által kódolt termék olyan szinten expresszálódik, ami terápiás hatást biztosít és ahol az alany korábban kapcsolatba került bármilyen AAV szerotípussal.
7. A 6. igénypont szerinti rekombináns AAV virion alkalmazásra, ahol a sejt vagy szöveti izomsejt vagy -szövet, és előnyösen vázizomból származik.
8. A 6. vagy 7. igénypont szerinti rekombináns AAV virion alkalmazásra, ahol a bejuttatás (i) intramuszkuláris injekcióval; (ii) a véráramba; (iii) intravénásan; (iv) intraarteriálisan; (v) a májba; vagy (vi) az agyba történik.
9. A 6-8. igénypontok bármelyike szerinti rekombináns AAV virion alkalmazásra, ahol a bejuttatás (i) intramuszkuláris injekcióval; (ii) a véráramba; vagy (iii) a májba történik.