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(54) Title: VIRUS VECTORS AND METHODS OF MAKING AND ADMINISTERING THE SAME

(57) Abstract

The present invention provides genetically–engineered parvovirus capsids and viruses designed to introduce a heterologous gene into a target cell. The parvoviruses of the invention provide a repertoire of vectors with altered antigenic properties, packaging capabilities, and/or cellular tropisms as compared with current AAV vectors.

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Virus Vectors and Methods of Making and Administering the Same

Related Application Information

This application claims the benefit of provisional applications Serial No. 60/107,840, filed on November 10, 1998, and Serial No. 60/123,651, filed on March 10, 1999, which are incorporated herein by reference in their entirety.

Statement of Federal Support

This invention was made, in part, with government support under grant numbers DK42701 and 5-32938 0-110 from the National Institutes of Health. The United States government has certain rights to this invention.

Field of the Invention

The present invention relates to virus vectors, in particular, modified parvovirus vectors and methods of making and administering the same.

Background

Parvoviruses are small, single-stranded, non-enveloped DNA viruses between twenty to thirty nanometers in diameter. The genomes of parvoviruses are approximately 5000 nucleotides long, containing two open reading frames. The left-hand open reading frame codes for the proteins responsible for replication (Rep), while the right-hand open reading frame encodes the structural proteins of the capsid (Cap). All parvoviruses have virions with icosahedral symmetry composed of a major Cap protein, usually the smallest of the Cap proteins, and one or two minor Cap proteins. The

Cap proteins are generated from a single gene that initiates translation from different start codons. These proteins have identical C-termini, but possess unique N-termini due to different initiation codons.

Most parvoviruses have narrow host ranges; the tropism of B19 is for human erythroid cells (Munshi *et al.*, (1993) *J. Virology* **67**:562), while canine parvovirus has a tropism for lymphocytes in adult dogs (Parrish *et al.*, (1988) *Virology* **166**:293; Chang *et al.*, (1992) J. *Virology* **66**:6858). Adeno-associated virus, on the other hand, can replicate well in canine, mouse, chicken, bovine, monkey, as well as numerous human lines, when the appropriate helper virus is present. In the absence of helper virus, AAV will infect and establish latency in all of these cell types, suggesting that the AAV receptor is common and conserved among species. Several serotypes of AAV have been identified, including serotypes 1, 2, 3, 4, 5 and 6.

Adeno-associated virus (AAV) is a dependent parvovirus twenty nanometers in size which requires co-infection with another virus (either adenovirus or certain members of the herpes virus group) to undergo a productive infection in cultured cells. In the absence of co-infection with helper virus, the AAV virion binds to a cellular receptor and enters the cell, migrating to the nucleus, and delivers a single-stranded DNA genome that can establish latency by integration into the host chromosome. The interest in AAV as a vector has centered around the biology of this virus. In addition to its unique life-cycle, AAV has a broad host range for infectivity (human, mouse, monkey, dog, *etc.*), is ubiquitous in humans, and is completely nonpathogenic. The finite packaging capacity of this virus (4.5kb) has restricted the use of this vector in the past to small genes or cDNAs. To advance the prospects of AAV gene delivery, vectors sufficient to carry larger genes must be developed. In addition, virions that specifically and efficiently target defined cell types without transducing others will be required for clinical application.

The capsid proteins of AAV2 are Vp1, 2, and 3 with molecular weights of 87, 73, and 62 kDa, respectively. Vp3 represents nearly 80% of the total protein in intact virions, while Vp1 and Vp2 represent 10% each (Muzyczka,

(1992) *Curr. Topics Microbiol. Immunol.* **158**:97; Rolling *et al.*, (1995) *Molec. Biotech.* **3**:9; Wistuba *et al.* (1997) *J. Virology* **71**:1341). Early studies of AAV2 support that all three capsid subunits are required to extract single stranded genomes from the pool of replicating double stranded DNA. These genomes are then sequestered into preformed immature particles that maturate to infectious particles. These particles have a density between 1.32 and 1.41g/mL in cesium chloride and sediment between 60S and 125S in sucrose (Myers *et al.*, (1981) *J. Biological Chem.* **256**:567; Myers *et al.*, (1980) *J. Virology* **35**:65).

Previous mutagenesis studies of AAV2 capsids have shown that insertions and deletions in the Vp3 domain completely inhibit the accumulation of single stranded virions and production of infectious particles (Hermonat *et al.*, (1984) *J. Virology* **51**:329; Ruffing *et al.*, (1992) *J. Virology* **66**:6922). Yang *et al.*, (1998) *Human Gene Therapy* **9**:1929, have reported the insertion of a sequence encoding the variable region of a single chain antibody against human CD34 at the 5' end of the AAV2 Vp1, Vp2 or Vp3 coding regions. These investigators observed extremely low transduction of CD34 expressing KG-1 cells by AAV virions containing the Vp2 fusion protein (1.9 transducing units/ml or less, sentence spanning pages1934-35). KG-1 cells are reportedly not permissive to infection by a wild-type rAAV vector. These results with the Vp2 fusion AAV are suspect as transduction of KG-1 cells by this virus was essentially insensitive to an anti-AAV capsid antibody (430 vs. 310 transducing units/ml; **Table 2**), whereas transduction of HeLa cells was markedly reduced by this antibody (63,2000 vs. <200 transducing units/ml; Table 2). No characterization of the putative fusion virions was undertaken to confirm that the particles contained the Vp2 fusion protein, the antibody was expressed on the capsid surface, or that the particles bound CD34 proteins. In addition, rAAV particles could only be produced if all three wild-type capsid subunits were provided, in addition to the chimeric subunit (Page 1934, Col. 2, lines 5-12). Collectively, these results suggest the chimeric subunits were not incorporated into viable AAV particles, and the low

level of chimeric protein observed in target cells was, in fact, due to cellular uptake of chimeric capsid protein or protein aggregates by other mechanisms.

Several studies have demonstrated that parvovirus capsid proteins can be mutated and virion assembly studied. In one study, the coding region for 147 amino acids of the hen egg white lysozyme was substituted for B19 Vp1 unique coding sequence. This modification resulted in purified empty particles that retained lysozyme enzymatic activity (Miyamura *et al.*, (1994) *Proc. Nat. Acad. Sci. USA* **91**:8507). In addition, expression of peptides (9 and 13 residues) in B19 Vp2 resulted in empty particles that were immunogenic in mice supporting surface presentation of the insertions (Brown *et al.*, (1994) *Virology* **198**:477). In a more recent study, the CD8+CTL epitope (residues 118-132) against lymphocytic choriomeningitis virus (LCMV) nucleoprotein was inserted into the Vp2 capsid protein of porcine parvovirus (ppv) (Sedlik *et al.*, (1997) *Proc. Nat. Acad. Sci. USA* **94**:7503). This capsid protein, with the epitope cloned at the N-terminus, self-assembled when expressed in a baculovirus system. This chimeric virus-like particle was then used to immunize mice against a lethal challenge from LCMV. While these studies evaluated capsid structure and assembly, they did not address the issue of packaging B19 genomes into the altered capsids.

Recombinant (r)AAV vectors require only the inverted terminal repeat sequences in *cis* of the 4679 bases to generate virus. All other viral sequences are dispensable and may be supplied in *trans* (Muzyczka, (1992) *Curr. Topics Microbiol. Immunol.* **158**:97). Attractive characteristics of AAV vectors for gene therapy are the stability, genetic simplicity, and ease of genetic manipulation of this virus. While each of these factors remains valid, some obstacles to the application of rAAV vectors have recently come to light. These include inefficiency of vector transduction and packaging constraints. It is not surprising, given the cryptic nature of this virus, that new insights into its biology have surfaced only after extensive research with rAAV vectors, which are more easily assayed compared with wild-type AAV.

With respect to the efficiency of vector transduction, several recent studies have shown great promise in terms of duration of transgene

expression *in vivo*; however, there has been a shortfall in the efficiency of transduction, which was unexpected based on previous results *in vitro* (Flotte *et al.*, (1993) *Proc. Nat. Acad. Sci. USA* **90**:10613). One of the first experiments in rodents to demonstrate the utility of rAAV vectors *in vivo* was aimed at transduction of brain tissue in rat (Kaplitt *et al.*, (1994) *Nature Genet.* **7**:148). In addition to brain, muscle has been found to be efficiently transduced *in vivo* by AAV vectors, demonstrating long term gene expression (at least 1.5 years), lack of immune response, and no vector toxicity (Xiao *et al.*, (1996) *J. Virol.* **70**:8098; Clark *et al.*, (1996) *Hum. Gene Ther.* **8**:659; Fisher *et al.*, (1997) *Nat. Med.* **3**:306; Monahan *et al.*, (1998) *Gene Ther.* **5**:40). The primary steps that influence efficient vector delivery are virus entry and conversion of second strand synthesis (*see* Ferrari *et al.*, (1996) *J. Virology* **70**:3227-34).

The overall success of AAV as a general-purpose viral vector depends on the ability to package larger than full-length AAV genomes (5 kb) into rAAV vectors. Studies by Dong *et al.*, (1996) *Hum. Gene Ther.* **7**:2101, have determined the packaging limitations using rAAV vectors as between 104% and 108%. This packaging restriction precludes the use of a number of important genes currently being tested for human gene therapy (*e.g.*, the dystrophin gene or current mini-dystrophin constructs).

Accordingly, there remains a need in the art for improved virus vectors with greater packaging limits and transduction efficiency than AAV vectors. In addition, there remains a need for virus vectors with altered tropisms as compared with AAV vectors.

Summary of the Invention

The present invention provides parvovirus vectors for introducing (*i.e.*, delivering) and, preferably, expressing a nucleotide sequence in a cell. The invention is based, in part, on the discovery that parvovirus vectors possessing unique structures and characteristics as compared with current vectors may be created by substituting or inserting a foreign sequence (*i.e.*, an exogenous amino acid sequence) into a parvovirus capsid. The invention

further provides novel vectors that are generated by cross-packaging a parvovirus genome (preferably, an AAV genome) within a different parvovirus capsid. The present invention provides a repertoire of novel parvovirus vectors that may possess unique and advantageous antigenic properties, packaging capabilities, and cellular tropisms as compared with current AAV vectors.

These and other aspects of the invention are set forth in more detail in the description of the invention below.

Brief Description of the Drawings

Figure 1 shows the insertional mutagenesis strategy for the AAV2 capsid. A cassette containing the Kan^r gene flanked by *EcoRV* and *Nae I* sites were cloned into the plasmid pAV2Cap. pAV2Cap, which contains the open reading frame of AAV2 capsid, was partially digested with *Hae III*, *Nla IV*, and *Rsa I* separately so that unit length products were isolated. The 43 positions of restriction sites for these enzymes are shown above the diagram of the capsid open reading frame. The position of the Kan^r insert was mapped by restriction enzyme digestion and in some cases sequenced. Once the position was determined the Kan^r gene was removed by *EcoRV* digestion, and the capsid domain subcloned into pACG. This strategy resulted in inserting a 12 base pair fragment, with half *Nae I* sites flanking a unique *Eco RV* site, into the respective *Hae III*, *Nla IV*, and *Rsa I* sites. The twelve base pairs code for four amino acids one of which is shown above the diagram of pACG2.

Figure 2 shows the expression of capsid proteins in cells transfected with wild-type and insertion mutant helper plasmids of pACG2. Cell lysates from 293 cells transfected with 1, H2285; 2, H2634; 3, H2690; 4, N2944; 5, H2944; 6, H3595; 7, H4047; 8, wild-type were analyzed by acrylamide gel electrophoresis and immunoblotting with the B1 monoclonal antibody and detected by peroxidase-conjugated secondary antibody. On the left of the

Western blot are the positions of the molecular weight standards and on the right are the positions of the major capsid protein, VP3 and the minor capsid proteins VP2 and VP1.

Figure 3 shows expression of a Lac Z transgene in cells infected with insertion mutant or wild-type virus. **Panel A**. Dot blot hybridization to the Lac Z transgene. Cell lysates of adenovirus infected 293 cells transfected with the insertion mutant or wild-type helper plasmids and the Lac Z transgene containing vector were subjected to cesium chloride isopycnic gradient. Fractions from the gradient were treated with DNase and RNase prior to dot blotting to remove unpackaged nucleic acids, fraction numbers are labeled above the dot blot. Fraction 1 has a density range of 1.377-1.41, fraction 2 has a density range of 1.39-1.435, and fraction 3 has a density range of 1.42-1.45. The β-galactosidase gene was used as the control template, to estimate particle numbers. Estimates of particle number where derived assuming 1μg of 1000bp DNA has 9.1×10^{11} molecules. **Panel B**. Infection of HeLa cells with 1.75×10^{8} particles from various insertion mutants and wild-type capsid containing the Lac Z transgene. Cells expressing the transgene appear blue when stained with X-gal.

Figure 4 shows characterization of the insertion mutants using electron microscopy. 200uL samples of each virus from peak fraction of gradient were dialyzed against 1xPBS +1mM $MgCl_2$ and speed-vac desiccated, then resuspended in 20uL of distilled H_2O. Samples were negative stained with 2% phosphotungstic acid. **Panel A**. rAAV2 with wild-type virion. Infectious insertion viruses H2690 (**Panel B**), and H2591 (**Panel C**). Non-infectious viruses H2285 (**Panel D**), H2634 (**Panel E**) and, H3595 (**Panel F**). The black bar is 100nm; the magnification is equivalent in each panel.

Figure 5 presents analysis of virion composition from wild-type and various insertion mutant viruses isolated from cell lysates by cesium chloride

gradient centrifugation. Peak fractions of virus were determined by dot blot hybridization and dialyzed against 1xPBS + 1mM $MgCl_2$. For each, viral sample between 1.0 $x10^9$ and 2.5 x 10^9 particles were used. Virions from 1. Wild-type rAAV2; 2. H2285; 3. R2349; 4. H2591; 5. H2634; 6. H2690; 7. H3766; and 8. N4160 were analyzed by acrylamide gel electrophoresis and immunoblotting with the B1 monoclonal antibody and detected by peroxidase-conjugated secondary antibody. On the left of the Western blot are the positions of the molecular weight standards and on the right are the positions of the major capsid protein, VP3 and the minor capsid proteins VP2 and VP1.

Figure 6 shows the analysis of wild-type and non-infectious insertion mutant virus batch binding to heparin agarose by dot blot hybridization. Viruses with wild-type virions and insertion in the capsids were dialysed against 0.5 x PBS and 0.5mM $MgCl_2$. One hundred microliters of each virus was bound to 100µl of heparin agarose, at room temperature for one hour. Samples were washed six times with 500µl of wash buffer each, followed by elution with 100µl of 0.5, 1.0 and 1.5M NaCl each, and the supernatant from each wash and elution step was saved. Twenty microliters of supernatant from each step and 20µl of the agarose pellet were used for dot blot hybridization. Pairs of washes were combined and 1/50 of the total volume from each pair was used for dot blot hybridization, while one fifth of the elution supernatant and agarose bed volumes were used. The 100% bound was equivalent to one fifth of the virus added to the heparin agarose. Samples 1. rAAV2 with wild-type virion; 2. H2285; 3. H2416; 4. H2634; and 5. H3761.

Figure 7 is schematic representation of the AAV2/4 subunit chimeras.

Figure 8 is a diagram of the helper plasmid pAAV2/B19p2Cap. The coding region of the B19 major structural protein, Vp2, was seamlessly cloned from AAV-Vp3 to TAA.

Figure 9 provides EM analysis of chimeric virus particles produced with pAAV/B19Vp2Cap.

Detailed Description of the Invention

The present invention provides parvovirus vectors for the delivery of nucleic acids to cells, both *in vitro* and *in vivo*. Alternatively, the invention provides novel capsid structures for use, *e.g.*, as vaccines or for delivery of compounds to cells (*e.g.*, as described by U.S. Patent No. 5,863,541 to Samulski *et al.*, the disclosure of which is incorporated herein by reference in its entirety). The parvovirus vectors of the present invention utilize the advantageous properties of AAV vectors, and may mitigate some of the problems encountered with these vectors. In particular embodiments, the parvovirus vectors may possess different or altered characteristics from AAV vectors, including but not limited to, antigenic properties, packaging capabilities, and/or cellular tropism.

The term "parvovirus" as used herein encompasses all parvoviruses, including autonomously-replicating parvoviruses and dependoviruses. The autonomous parvoviruses include members of the genera *Parvovirus*, *Erythrovirus*, *Densovirus*, *Iteravirus*, and *Contravirus*. Exemplary autonomous parvoviruses include, but are not limited to, mouse minute virus, bovine parvovirus, canine parvovirus, chicken parvovirus, feline panleukopenia virus, feline parvovirus, goose parvovirus, and B19 virus. Other autonomous parvoviruses are known to those skilled in the art. *See, e.g.*, BERNARD N. FIELDS *et al.*, VIROLOGY, volume 2, chapter 69 (3d ed., Lippincott-Raven Publishers).

The genus *Dependovirus* contains the adeno-associated viruses (AAV), including but not limited to, AAV type 1, AAV type 2, AAV type 3, AAV type 4, AAV type 5, AAV type 6, avian AAV, bovine AAV, canine AAV, equine AAV, and ovine AAV. *See, e.g.*, BERNARD N. FIELDS *et al.*, VIROLOGY, volume 2, chapter 69 (3d ed., Lippincott-Raven Publishers).

The parvovirus particles, capsids and genomes of the present invention are preferably from AAV.

The term "tropism" as used herein refers to entry of the virus into the cell, optionally and preferably, followed by expression of sequences carried by the viral genome in the cell, *e.g.*, for a recombinant virus, expression of the heterologous nucleotide sequences(s). Those skilled in the art will appreciate that transcription of a heterologous nucleic acid sequence from the viral genome may not be initiated in the absence of trans-acting factors, *e.g.*, for an inducible promoter or otherwise regulated nucleic acid sequence. In the case of AAV, gene expression from the viral genome may be from a stably integrated provirus, from a non-integrated episome, as well as any other form in which the virus may take within the cell.

The parvovirus vectors of the present invention are useful for the delivery of nucleic acids to cells both *in vitro* and *in vivo*. In particular, the inventive vectors may be advantageously employed to deliver or transfer nucleic acids to animal cells. Nucleic acids of interest include nucleic acids encoding peptides and proteins, preferably therapeutic (*e.g.*, for medical or veterinary uses) or immunogenic (*e.g.*, for vaccines) peptides or proteins.

A "therapeutic" peptide or protein is a peptide or protein that may alleviate or reduce symptoms that result from an absence or defect in a protein in a cell or subject. Alternatively, a "therapeutic" peptide or protein is one that otherwise confers a benefit to a subject, *e.g.*, anti-cancer effects. Therapeutic peptides and proteins include, but are not limited to, CFTR (cystic fibrosis transmembrane regulator protein), dystrophin (including the protein product of dystrophin mini-genes, *see, e.g*, Vincent *et al.*, (1993) *Nature Genetics* **5**:130), utrophin (Tinsley *et al.*, (1996) *Nature* **384**:349), clotting factors (Factor XIII, Factor IX, Factor X, *etc.)*, erythropoietin, the LDL receptor, lipoprotein lipase, ornithine transcarbamylase, β-globin, α-globin, spectrin, α-antitrypsin, adenosine deaminase, hypoxanthine guanine phosphoribosyl transferase, β-glucocerebrosidase, sphingomyelinase, lysosomal hexosaminidase, branched-chain keto acid dehydrogenase, hormones, growth factors (*e.g.*, insulin-like growth factors 1 and 2, platelet derived growth factor, epidermal growth factor, nerve growth factor, neurotrophic factor –3 and –4, brain-derived neurotrophic factor, glial derived

growth factor, transforming growth factor-α and –β, and the like), cytokines (*e.g.*, α-interferon, β-interferon, interferon-γ, interleukin-2, interleukin-4, interleukin 12, granulocyte-macrophage colony stimulating factor, lymphotoxin), suicide gene products (*e.g.*, herpes simplex virus thymidine kinase, cytosine deaminase, diphtheria toxin, cytochrome P450, deoxycytidine kinase, and tumor necrosis factor), proteins conferring resistance to a drug used in cancer therapy, tumor suppressor gene products (*e.g.*, p53, Rb, Wt-1, NF1, VHL, APC, and the like), and any other peptide or protein that has a therapeutic effect in a subject in need thereof.

Further exemplary therapeutic peptides or proteins include those that may used in the treatment of a disease condition including, but not limited to, cystic fibrosis (and other diseases of the lung), hemophilia A, hemophilia B, thalassemia, anemia and other blood disorders, AIDS, Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, epilepsy, and other neurological disorders, cancer, diabetes mellitus, muscular dystrophies (*e.g.*, Duchenne, Becker), Gaucher's disease, Hurler's disease, adenosine deaminase deficiency, glycogen storage diseases and other metabolic defects, retinal degenerative diseases (and other diseases of the eye), and diseases of solid organs (*e.g.*, brain, liver, kidney, heart).

The present invention also provides vectors useful as vaccines. The use of parvoviruses as vaccines is known in the art (*see, e.g.*, Miyamura *et al.*, (1994) *Proc. Nat. Acad. Sci USA* **91**:8507; U.S. Patent No. 5,916,563 to Young *et al.*, 5,905,040 to Mazzara *et al.*, U.S. Patent No. 5,882,652, U.S. Patent No. 5,863,541 to Samulski *et al.*; the disclosures of which are incorporated herein in their entirety by reference). The antigen may be presented in the parvovirus capsid, as described below for chimeric and modified parvovirus vectors. Alternatively, the antigen may be expressed from a heterologous nucleic acid introduced into a recombinant AAV genome and carried by the inventive parvoviruses. Any immunogen of interest may be provided by the parvovirus vector. Immunogens of interest are well-known in the art and include, but are not limited to, immunogens from human

immunodeficiency virus, influenza virus, gag proteins, tumor antigens, cancer antigens, bacterial antigens, viral antigens, and the like.

As a further alternative, the heterologous nucleic acid sequence may encode a reporter peptide or protein (*e.g.*, an enzyme). Reporter proteins are known in the art and include, but are not limited to, Green Fluorescent Protein, β-galactosidase, alkaline phosphatase, chloramphenicol acetyltransferase, and the like.

Alternatively, in particular embodiments of the invention, the nucleic acid of interest may encode an antisense nucleic acid, a ribozyme (*e.g.*, as described in U.S. Patent No. 5,877,022), RNAs that effect spliceosome-mediated *trans*-splicing (Puttaraju *et al.*, (1999) *Nature Biotech.* **17**:246), or other non-translated RNAs, such as "guide" RNAs (Gorman *et al.*, (1998) *Proc. Nat. Acad. Sci. USA* **95**:4929; U.S. Patent No. 5,869,248 to Yuan *et al.*), and the like.

Except as otherwise indicated, standard methods known to those skilled in the art may be used for the construction of rAAV genomes, transcomplementing packaging vectors, transiently and stably transfected packaging cells according to the present invention. Such techniques are known to those skilled in the art. *See, e.g.*, SAMBROOK *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL 2nd Ed. (Cold Spring Harbor, NY, 1989); F. M. AUSUBEL *et al.* CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Green Publishing Associates, Inc. and John Wiley & Sons, Inc., New York).

I. Hybrid Viruses.

The hybrid parvovirus vectors of the present invention may overcome some of the disadvantages of AAV vectors for delivery of nucleic acids or other molecules to cells.

A "hybrid" parvovirus, as used herein, is an AAV genome encapsidated within a different (*i.e.*, another, foreign, exogenous) parvovirus capsid. Alternatively stated, a hybrid parvovirus has a parvovirus genome encapsidated within a different parvovirus capsid. As used herein, by "different" it is intended that the AAV genome is packaged within another

parvovirus capsid, *e.g.*, the parvovirus capsid is from another AAV serotype or from an autonomous parvovirus.

Preferably, the parvovirus genome is an AAV genome (preferably a recombinant AAV genome). It is also preferred that the AAV genome comprises one or more AAV inverted terminal repeat(s) as described below. Typically, as described in more detail below, a recombinant AAV (rAAV) genome will retain only those elements required in *cis* (*e.g.*, one or more AAV ITRs), with the rest of the genome (*e.g.*, the rep/cap genes) being provided in *trans*.

In particular preferred embodiments the parvovirus capsid is an AAV capsid (*i.e.*, a hybrid AAV vector). According to this embodiment, the AAV capsid packages an AAV genome of a different serotype (and preferably, of a different serotype from the one or more AAV ITRs). For example, a recombinant AAV type 1, 2, 3, 4, 5 or 6 genome may be encapsidated within an AAV type 1, 2, 3, 4, 5 or 6 capsid, provided that the AAV capsid and genome (and preferably, the one or more AAV ITRs) are of different serotypes.

Illustrative hybrid parvoviruses according to the present invention are an AAV type 2 genome packaged within an AAV type 1, 3, 4, 5 or 6 capsid. In particular preferred embodiments, the hybrid parvovirus comprises an AAV type 3, type 4, or type 5 capsid packaging an AAV type 2 genome, more preferably, an AAV type 3 or type 5 capsid packaging a type 2 genome.

In other preferred embodiments, an AAV type 1, 3, 4, 5 or 6 genome is packaged within a different AAV capsid (*e.g.*, a type 1 genome in a type 2, 3, 4, 5, or 6 capsid, and the like).

Also preferred are hybrid B19/AAV parvoviruses in which an AAV genome (*e.g.*, an AAV type 1, 2, 3, 4, 5 or 6 genome) is packaged within a B19 capsid. More preferably, the hybrid parvovirus has a B19 capsid and an AAV type 2 genome.

Further preferred are hybrid parvoviruses in which a mouse minute virus, bovine parvovirus, canine parvovirus, chicken parvovirus, feline

panleukopenia virus, feline parvovirus, or goose parvovirus capsid packages an AAV genome, more preferably an AAV type 2 genome.

Specific hybrid viruses include those having the capsid sequence encoded by the AAV2/4 helper plasmid given in **Appendix 1** (nucleotides 2123 to 4341 of **SEQ ID NO:1**). This sequence encodes the AAV2 rep genes and AAV4 capsid in a pBluescript backbone. It is also preferred that the hybrid parvovirus having the capsid sequence given by **SEQ ID NO:1** is an AAV2 genome. Alternatively, the nucleotide sequence of the AAV4 capsid is substantially homologous to the nucleotide sequence given as nucleotides 2123 to 4341 of **SEQ ID NO:1**. As a further alternative, the nucleotide sequence of the AAV4 capsid encodes the amino acid sequence encoded by nucleotides 2123 to 4341 in **SEQ ID NO:1**. The term "substantially homologous" is as defined hereinbelow.

One of the limitations of current AAV vectors for gene delivery is the prevalence of neutralizing antibodies against AAV within the human population. For example, it is estimated that 80% of adults are seropositive for AAV type 2. In preferred embodiments, the instant invention provides hybrid parvovirus vectors that may be advantageously employed to reduce (*e.g.*, diminish, decrease, mitigate, and the like) an immune response in the subject being treated. Thus, for example, a rAAV type 2 vector genome carrying a heterologous nucleic acid sequence or sequences may be packaged within an AAV type 3 capsid and administered to a subject who is seropositive for AAV type 2 and cannot neutralize AAV type 3 virus.

According to this aspect of the invention, a rAAV genome may be packaged within any non-homologous parvovirus capsid for delivery to a cell, *in vitro* or *in vivo*. In preferred embodiments, the AAV genome is packaged within an array of non-homologous capsids to overcome neutralizing antibodies and/or or to prevent the development of an immune response. In particular preferred embodiments, the rAAV may be delivered within a series of hybrid virus particles, so as to continually present the immune system with a new virus vector. This strategy will allow for repeated administration without immune clearance.

A further limitation encountered with AAV vectors concerns the cellular tropism of this virus. The wild-type tropism of AAV is problematic both because AAV infects a wide range of cell types and because it exhibits no infectivity in other potential target cells of interest (*e.g*, erythroid cells). Autonomous parvoviruses, in contrast, have a narrower cellular tropism. The tropisms of particular autonomous parvoviruses are known to those skilled in the art. Illustrative cellular tropisms of autonomous parvoviruses include: B19 virus (erythroid cells), canine parvovirus (gut epithelium), MVM(p) (fibroblasts); and goose parvovirus (myocardial lining of the heart). Furthermore, autonomous parvoviruses exhibit a wider range of host species than does AAV, which characteristic may be utilized to develop AAV vectors for administration to bovines, canines, felines, geese, ducks, and the like, *e.g.*, for veterinary treatments. Thus, cross-packaging of AAV genomes in autonomous parvovirus capsids according to the present invention may be utilized to produce a virus vector with a different cellular tropism than AAV.

With respect to AAV/AAV hybrids, all of the AAV serotypes infect a broad host range of cells. However, there are differences in the rates of vector transduction, suggesting that the different serotypes may use different cellular receptors. In addition, only limited competition is observed among serotypes in binding experiments, which observation further indicates that the different serotypes have evolved to use distinct receptors (Mizukami *et al.*, (1996) *Virology* **217**:124). Accordingly, hybrid parvoviruses of the present invention that package an AAV genome in an AAV capsid of a different serotype also provide opportunities for delivering AAV vectors to a wider range of cell types than current AAV vectors and/or for directing AAV vectors to specific target cells.

In preferred embodiments, the hybrid parvovirus particle contains a rAAV genome. As used herein, the rAAV genome carries at least one heterologous nucleic acid sequence to be delivered to a cell. Those skilled in the art will appreciate that the rAAV genome can encode more than one heterologous nucleic acid sequence (*e.g.*, two, three or more heterologous nucleic acid sequences), generally only limited by the packaging capacity of

the virus capsid. Heterologous nucleic acid sequence(s) of interest for use according to the present invention are as described above.

As used herein, a recombinant hybrid parvovirus particle encompasses virus particles with hybrid, chimeric, targeted and/or modified parvovirus capsids as described hereinbelow. Moreover, those skilled in the art will understand that the parvovirus capsid may include other modifications or mutations (*e.g.*, deletion, insertion, point and/or missense mutations, and the like). Likewise, the rAAV genome may include modifications or mutations (*e.g.*, deletion, insertion, point and/or missense mutations, and the like). Those skilled in the art will further appreciate that mutations may incidentally be introduced into the rAAV genome or parvovirus capsid as a result of the cloning strategy employed.

The rAAV genome of the hybrid parvovirus preferably encodes at least one AAV inverted terminal repeat (ITR), preferably two AAV ITRs, and more preferably two homologous AAV ITRs, which flank the heterologous nucleic acid sequence(s) to be delivered to the cell. The AAV ITR(s) may be from any AAV, with types 1, 2, 3, 4, 5 and 6 being preferred, and type 2 being most preferred. The term "inverted terminal repeat" includes synthetic sequences that function as an AAV inverted terminal repeat, such as the "double-D sequence" as described in United States Patent No. 5,478,745 to Samulski *et al.*, the disclosure of which is incorporated in its entirety herein by reference. It has been demonstrated that only a single 165 bp double-D sequence is required in *cis* for site specific integration, replication, and encapsidation of vector sequences. AAV ITRs according to the present invention need not have a wild-type ITR sequence (*e.g.*, a wild-type sequence may be altered by insertion, deletion, truncation or missense mutations), as long as the ITR functions to mediate virus packaging, replication, integration, and/or provirus rescue, and the like.

In hybrid parvoviruses according to the present invention, the AAV ITR(s) is different from the parvovirus capsid. Moreover, if the capsid is an AAV capsid, the capsid and the ITR(s) are of different AAV serotypes. In preferred embodiments, the AAV ITR(s) is from AAV type 2 and the

parvovirus capsid is an AAV type 3, 4 or 5 capsid, more preferably an AAV type 3 or 5 capsid. In alternate preferred embodiments, the hybrid parvovirus has a B19 capsid and the AAV ITR(s) is from AAV type 2.

The rAAV genomes of the invention may additionally contain expression control elements, such as transcription/translation control signals, origins of replication, polyadenylation signals, and internal ribosome entry sites (IRES), promoters, enhancers, and the like, operably associated with the heterologous nucleic acid sequence(s) to be delivered to the cell. Those skilled in the art will appreciate that a variety of promoter/enhancer elements may be used depending on the level and tissue-specific expression desired. The promoter/enhancer may be constitutive or inducible, depending on the pattern of expression desired. The promoter/enhancer may be native or foreign and can be a natural or a synthetic sequence. By foreign, it is intended that the promoter/enhancer region is not found in the wild-type host into which the promoter/enhancer region is introduced.

Promoters/enhancers that are native to the target cell or subject to be treated are most preferred. Also preferred are promoters/enhancers that are native to the heterologous nucleic acid sequence. The promoter/enhancer is chosen so that it will function in the target cell(s) of interest. Mammalian promoters/enhancers are also preferred.

Inducible expression control elements are preferred in those applications in which it is desirable to provide regulation over expression of the heterologous nucleic acid sequence(s). Inducible promoters/enhancer elements for gene delivery are preferably tissue-specific promoter/enhancer elements, and include muscle specific (including cardiac, skeletal and/or smooth muscle), neural tissue specific (including brain-specific), liver specific, bone marrow specific, pancreatic specific, spleen specific, retinal specific, and lung specific promoter/enhancer elements. Other inducible promoter/enhancer elements include hormone-inducible and metal-inducible elements. Exemplary inducible promoters/enhancer elements include, but are not limited to, a Tet on/off element, a RU486-inducible promoter, an

ecdysone-inducible promoter, a rapamycin-inducible promoter, and a metalothionein promoter.

In embodiments of the invention in which the heterologous nucleic acid sequence(s) will be transcribed and then translated in the target cells, specific initiation signals are generally required for efficient translation of inserted protein coding sequences. These exogenous translational control sequences, which may include the ATG initiation codon and adjacent sequences, can be of a variety of origins, both natural and synthetic.

The AAV genome of the inventive parvovirus vectors may optionally include the genes that encode the AAV Cap and Rep proteins. In preferred embodiments, the genes encoding at least one of the AAV Cap proteins or at least one of the AAV Rep proteins will be deleted from the rAAV genome. According to this embodiment, the Cap and Rep functions may be provided *in trans*, *e.g.*, from a transcomplementing packaging vector or by a stably-transformed packaging cell line. In more preferred embodiments, the genes encoding all of the AAV Cap proteins or all of the AAV Rep proteins will be deleted from the rAAV genome. Finally, in the most preferred embodiments, all of the AAV cap genes and all of the AAV rep genes are deleted from the AAV vector. This configuration maximizes the size of the heterologous nucleic acid sequence(s) that can be carried by the AAV genome, simplifies cloning procedures, and minimizes recombination between the rAAV genome and the rep/cap packaging sequences provided in *trans*.

In hybrid parvoviruses according to the present invention, the parvovirus cap genes (if present) may encode the Cap proteins from any parvovirus, preferably an AAV. In contrast, the rep genes (if present) will typically and preferably be AAV rep genes. It is further preferred that the rep genes and the AAV inverted terminal repeat(s) carried by the AAV genome are of the same serotype. Moreover, if the cap genes are AAV cap genes, the rep genes will preferably be of a different AAV serotype from the AAV cap genes.

The rep genes/proteins of different AAV serotypes may be evaluated for those giving the highest titer vector in connection with particular hybrid

parvoviruses without undue experimentation. In particular preferred embodiments, the AAV rep genes encode a temperature-sensitive Rep78 and/or Rep68 protein as described by Gavin *et al.*, (1999) *J. Virology* **73**:9433 (the disclosure of which is incorporated herein by reference in its entirety).

As described above, the Cap proteins of the hybrid parvovirus are different from the AAV genome (*i.e.*, the Cap proteins are either from a different AAV serotype or from an autonomous parvovirus). In addition, as described above, the Cap proteins will typically and preferably be different from the rep genes (if present).

Accordingly, in particular preferred embodiments, the hybrid parvovirus has an AAV type 3, 4 or 5 capsid and carries an AAV type 2 genome including an AAV type 2 ITR(s). The AAV genome may additionally include the AAV rep genes (preferably type 2) and AAV cap genes (preferably, AAV type 3, 4, or 5, respectively). Typically, however, the AAV genome will be a rAAV genome, and the rep and cap genes will be deleted therefrom. In an alternate preferred embodiment, the hybrid parvovirus has a B19 capsid and carries an AAV genome, more preferably an AAV type 2 genome, including an AAV ITR(s). The AAV genome may optionally encode the AAV Rep proteins (preferably AAV type 2) and B19 capsid proteins, but preferably is a rAAV genome lacking these sequences.

The present invention also provides nucleotide sequences and vectors (including cloning and packaging vectors) encoding the inventive AAV genomes and the parvovirus cap gene(s) and the AAV rep gene(s) for producing the inventive hybrid parvoviruses. As described above, in preferred embodiments, at least one of the AAV rep genes or one of the AAV cap genes, more preferably all of the AAV rep genes and the AAV cap genes, are deleted from the AAV genome. The Rep and Cap functions may be provided *in trans* by packaging vector(s). Multiple packaging vectors (*e.g.*, two, three, *etc.*) may be employed, but typically and preferably all of the Rep and Cap functions are provided by a single packaging vector.

Cloning and packaging vectors may be any vector known in the art. Illustrative vectors include, but are not limited to, plasmids, naked DNA

vectors, bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs), and viral vectors. Preferred viral vectors include AAV, adenovirus, herpesvirus, Epstein-Barr virus (EBV), baculovirus, and retroviral (*e.g.*, lentiviral) vectors, more preferably, adenovirus and herpesvirus vectors.

The present invention also provides cells containing the inventive vectors. The cell may be any cell known in the art including bacterial, protozoan, yeast, fungus, plant, and animal (*e.g.*, insect, avian, mammalian) cells.

Further provided are stably-transformed packaging cells that express the sequences encoding the parvovirus cap gene(s) and/or the AAV rep gene(s) for producing the inventive hybrid parvoviruses. Any suitable cell known in the art may be employed to express the parvovirus cap and/or rep gene(s). Mammalian cells are preferred (*e.g.*, HeLa cells). Also preferred are trans-complementing packaging cell lines that will provide functions deleted from a replication-defective helper virus, *e.g.*, 293 cells or other E1a trans-complementing cells.

In particular preferred embodiments, at least one of the rep genes or at least one of the cap genes, more preferably all of the cap genes or all of the rep genes are stably integrated into the genetic material of the packaging cell and are expressed therefrom. Typically, and most preferably, all of the parvovirus cap genes and all of the AAV rep genes are stably integrated and expressed by the packaging cell.

The cap and rep genes and proteins are as described above with respect to hybrid AAV genomes. Thus, the packaging vector(s) and/or packaging cell may encode the cap genes from any parvovirus. Preferred are the B19, AAV type 3, AAV type 4 and AAV type 5 cap genes. Likewise, the packaging vector(s) and/or packaging cell may encode the rep genes from any parvovirus. Preferably, however, the rep genes will be AAV genes, more preferably, AAV type 2, AAV type 3, AAV type 4, or AAV type 5 rep genes. Most preferably, the rep genes are AAV type 2 rep genes. In particular preferred embodiments, the AAV rep sequences encode a temperature-

sensitive Rep78 or Rep68 protein as described by Gavin *et al.*, (1999) *J. Virology* **73**:9433.

The expression of the cap and rep genes, whether carried by the rAAV genome, a packaging vector, or stably integrated into the genome of a packaging cell may be driven by any promoter or enhancer element known in the art, as described in more detail above. Preferably, the cap or rep genes (more preferably both) are operably associated with parvovirus promoters. In the most preferred embodiments, the cap genes and rep genes are operably associated with their authentic promoters (*i.e.*, the native promoter).

A previous report indicates that expression of parvovirus cap genes from a B19/AAV type 2 hybrid helper vector cannot be achieved using authentic promoters. Ponnazhagan *et al.*, (1998) *J. Virology* **72**:5224, attempted to generate a helper vector for producing a B19 parvovirus capsid packaging an AAV type 2 genome. These investigators reported that virus could not be packaged when the cap genes on the helper vector were driven by either the authentic AAV p40 or B19 p6 promoters. Packaging of virus was only successfully achieved when the CMV promoter (a strong promoter) was substituted for the authentic promoters. It appears that the natural regulation of the *cap* genes was disrupted, and *cap* gene expression was restored only by splitting up the *rep* and *cap* coding regions and using an exogenous promoter to drive *cap* gene expression.

Likewise, the cloning strategy proposed by U.S. Patent No. 5,681,731 to Lebkowski *et al.* for generating hybrid viruses comprising an autonomous parvovirus capsid encapsidating a rAAV genome (col. 15-16) will fail to result in packaged virus.

In contrast, the present invention provides hybrid packaging vectors and packaging cells in which parvovirus promoters, preferably the authentic promoters, may be used to drive expression of the parvovirus cap and rep genes to produce the inventive hybrid parvoviruses. Previous efforts to create hybrid parvovirus cap/rep gene constructs using authentic promoters have not succeeded, at least in part, because these investigators failed to preserve the integrity of the splice sites required for proper processing of the

rep genes. The present investigations have utilized a seamless cloning strategy (Stratagene USA) in which the splice sites within the rep genes have been preserved. Alternatively, site-directed mutagenesis (or similar techniques) may be used to restore the splice sites to the hybrid virus constructs.

The present invention further encompasses methods of producing the inventive hybrid parvoviruses. Hybrid parvovirus particles according to the invention may be produced by introducing an AAV genome to be replicated and packaged into a permissive or packaging cell, as those terms are understood in the art (*e.g.*, a "permissive" cell can be infected or transduced by the virus; a "packaging" cell is a stably transformed cell providing helper functions). Preferably, the AAV genome is a rAAV genome encoding a heterologous nucleic acid sequence(s) that is flanked by at least one AAV ITR. rAAV genomes, AAV ITRs, and heterologous nucleic acid sequences are all as described in more detail hereinabove. The AAV genome may be provided to the cell by any suitable vector, as described hereinabove.

Any method of introducing the vector carrying the AAV genome into the permissive cell may be employed, including but not limited to, electroporation, calcium phosphate precipitation, microinjection, cationic or anionic liposomes, and liposomes in combination with a nuclear localization signal. In embodiments wherein the AAV genome is provided by a virus vector, standard methods for producing viral infection may be used.

Any suitable permissive or packaging cell known in the art may be employed to produce AAV vectors. Mammalian cells are preferred. Also preferred are trans-complementing packaging cell lines that provide functions deleted from a replication-defective helper virus, *e.g.*, 293 cells or other E1a trans-complementing cells.

The AAV genome may contain some or all of the AAV cap and rep genes, as described herein. Preferably, however, some or all of the cap and rep functions are provided *in trans* by introducing a packaging vector(s), as described above, into the cell. Alternatively, the cell is a packaging cell that is

stably transformed to express the cap and/or rep genes. Packaging vectors and packaging cells are as described hereinabove.

In addition, helper virus functions are provided for the AAV vector to propagate new virus particles. Both adenovirus and herpes simplex virus may serve as helper viruses for AAV. *See, e.g.*, BERNARD N. FIELDS *et al.*, VIROLOGY, volume 2, chapter 69 (3d ed., Lippincott-Raven Publishers). Exemplary helper viruses include, but are not limited to, Herpes simplex (HSV) varicella zoster, cytomegalovirus, and Epstein-Barr virus. The multiplicity of infection (MOI) and the duration of the infection will depend on the type of virus used and the packaging cell line employed. Any suitable helper vector may be employed. Preferably, the helper vector(s) is a plasmid, for example, as described by Xiao *et al.*, (1998) *J. Virology* **72**:2224. The vector can be introduced into the packaging cell by any suitable method known in the art, as described above.

AAV vectors can be produced by any suitable method known in the art. The traditional production of rAAV vectors entails co-transfection of a rep/cap vector encoding AAV helper and the AAV vector into human cells infected with adenovirus (Samulski *et al.*, (1989) *J. Virology* **63**:3822). Under optimized conditions, this procedure can yield up to 10^9 infectious units of rAAV per ml. One drawback of this method, however, is that it results in the co-production of contaminating wild-type adenovirus in rAAV preparations. Since several adenovirus proteins (*e.g.*, fiber, hexon, *etc.*) are known to produce a cytotoxic T-lymphocyte (CTL) immune response in humans (Yang and Wilson, (1995) *J. Immunol.* **155**:2564; Yang *et al.*, (1995) *J. Virology* **69**:2004; Yang *et al.*, (1994) *Proc. Nat. Acad. Sci. USA* **91**:4407), this represents a significant drawback when using these rAAV preparations (Monahan *et al.*, (1998) *Gene Therapy* **5**:40).

AAV vector stocks free of contaminating helper virus may be obtained by any method known in the art. For example, AAV and helper virus may be readily differentiated based on size. AAV may also be separated away from helper virus based on affinity for a heparin substrate (Zolotukhin *et al.* (1999) *Gene Therapy* **6**:973). Preferably, deleted replication-defective helper viruses

are used so that any contaminating helper virus is not replication competent. As a further alternative, an adenovirus helper lacking late gene expression may be employed, as only adenovirus early gene expression is required to mediate packaging of AAV virus. Adenovirus mutants defective for late gene expression are known in the art (*e.g.*, ts100K and ts149 adenovirus mutants).

A preferred method for providing helper functions through infectious adenovirus employs a non-infectious adenovirus miniplasmid that carries all of the helper genes required for efficient AAV production (Ferrari *et al.*, (1997) *Nature Med.* **3**:1295; Xiao *et al.*, (1998) *J. Virology* **72**:2224). The rAAV titers obtained with adenovirus miniplasmids are forty-fold higher than those obtained with conventional methods of wild-type adenovirus infection (Xiao *et al.*, (1998) *J. Virology* **72**:2224). This approach obviates the need to perform co-transfections with adenovirus (Holscher *et al.*, (1994), *J. Virology* **68**:7169; Clark *et al.*, (1995) *Hum. Gene Ther.* **6**:1329; Trempe and Yang, (1993), *in*, *Fifth Parvovirus Workshop*, Crystal River, FL).

Other methods of producing rAAV stocks have been described, including but not limited to, methods that split the *rep* and *cap* genes onto separate expression cassettes to prevent the generation of replication-competent AAV (*see, e.g.*, Allen *et al.*, (1997) *J. Virol.* **71**:6816), methods employing packaging cell lines (*see, e.g.*, Gao *et al.*, (1998) *Human Gene Therapy* **9**:2353; Inoue *et al.*, (1998) *J. Virol.* **72**:7024), and other helper virus free systems (*see*, *e.g.*, U.S. Patent No. 5,945,335 to Colosi).

Accordingly, the AAV genome to be packaged, parvovirus cap genes, AAV rep genes, and helper functions are provided to a cell (*e.g.*, a permissive or packaging cell) to produce AAV particles carrying the AAV genome. The combined expression of the rep and cap genes encoded by the AAV genome and/or the packaging vector(s) and/or the stably transformed packaging cell results in the production of a hybrid parvovirus in which a parvovirus capsid encapsidates an AAV genome. The hybrid parvovirus particles are allowed to assemble within the cell, and are then recovered by any method known by those of skill in the art.

The reagents and methods disclosed herein may be employed to produce high-titer stocks of the inventive parvovirus vectors. Preferably, the parvovirus stock has a titer of at least about 10^5 transducing units (tu)/ml, more preferably at least about 10^6 tu/ml, more preferably at least about 10^7 tu/ml, yet more preferably at least about 10^8 tu/ml, yet more preferably at least about 10^9 tu/ml, still yet more preferably at least about 10^{10} tu/ml, still more preferably at least about 10^{11} tu/ml, or more.

Alternatively stated, the parvovirus stock preferably has a titer of at least about 1 tu/cell, more preferably at least about 5 tu/cell, still more preferably at least about 20 tu/cell, yet more preferably at least about 50 tu/cell, still more preferably at least about 100 tu/cell, more preferably still at least about 250 tu/cell, most preferably at least about 500 tu/cell, or even more.

It is also preferred that the parvovirus is produced at essentially wild-type titers.

Those skilled in the art will appreciate that the instant invention also encompasses hybrid parvovirus vectors that contain chimeric capsids and/or capsids that have been modified by insertion of an amino acid sequence(s) into the capsid to confer altered tropisms or other characteristics, each as discussed in more detail below. The virus capsids may also include other modifications, *e.g.*, deletion, insertion, point and/or missense mutations, and the like.

Those skilled in the art will further appreciate that mutations may incidentally be introduced into the cap and/or rep genes as a result of the particular cloning strategy employed. For example, the construction of sequences encoding hybrid parvovirus genomes as described above may result in chimeric rep genes (and proteins) because of the overlap of the rep and cap sequences (*e.g.*, the cap genes and 3' end of the rep genes may be AAV type 3, and the remainder of the rep genes may be AAV type 2). As described above, chimeric AAV rep genes in which the 3' region is derived from an autonomous parvovirus will generally not function as the splicing signals are not conserved among AAV and the autonomous parvoviruses,

unless site-directed mutagenesis, or a similar technique, is employed to restore the splice sites to the hybrid virus constructs.

II. Chimeric Viruses.

The present invention further provides the discovery that chimeric parvoviruses may be constructed that possess unique capsid structures and characteristics. The strategy described above focused on altering AAV virus structure and function by cross-packaging AAV genomes within different parvovirus capsids. Further diversity in virus particles may be achieved by substituting a portion of the parvovirus capsid with a portion of a capsid(s) from a different (*i.e.*, another or foreign) parvovirus(es). Alternatively, a portion of a different parvovirus capsid(s) may be inserted (*i.e.*, rather than substituted) into the parvovirus capsid to create a chimeric parvovirus capsid. Also disclosed are vectors, packaging cells, and methods for constructing chimeric parvovirus particles. The chimeric parvoviruses disclosed herein may possess new antigenic properties, packaging capabilities, and/or cellular tropisms. The chimeric capsids and virus particles of the invention are also useful for raising chimera-specific antibodies against the novel capsid structures.

Parvoviruses, AAV, and rAAV genomes are as described above with respect to hybrid parvoviruses.

As used herein, a "chimeric" parvovirus is a parvovirus in which a foreign (i.e., exogenous) capsid region(s) from a different parvovirus(s) is inserted or substituted into the parvovirus capsid. Preferably the foreign capsid region is substituted for one of the native parvovirus capsid regions. In particular embodiments, the foreign capsid region is swapped for the homologous capsid region within the parvovirus capsid. It is also preferred that the parvovirus capsid is an AAV capsid. According to this embodiment, the AAV capsid may be of any AAV serotype (*e.g.*, type 1, type 2, type 3, type 4, type 5, type 6, *etc.*, as described above). More preferably, the AAV capsid is an AAV type 2, type 3, type 4, or type 5 capsid, most preferably an AAV type 2 capsid.

Those skilled in the art will appreciate that the chimeric parvovirus may additionally be a hybrid parvovirus (as described above) or may be a targeted, or otherwise modified, parvovirus (as described below). Those skilled in the art will further appreciate that due to the overlap in the sequences encoding the parvovirus capsid proteins, a single insertion or substitution may affect more than one capsid subunit.

The foreign parvovirus capsid region may be from any parvovirus (*i.e.*, an autonomous parvovirus or dependovirus) as described above. Preferably, the foreign capsid region is from the human B19 parvovirus or from AAV type 3, type 4, or type 5.

The foreign parvovirus capsid region may constitute all or substantially all of a capsid subunit(s) (*i.e.*, domain, for example the Vp1, Vp2 and Vp3 subunits of AAV or the Vp1 and Vp2 subunits of B19 virus) or a portion of a capsid subunit. Conversely, more than one foreign capsid subunit may be inserted or substituted into the parvovirus capsid. Likewise, a portion of a parvovirus capsid subunit or one or more parvovirus capsid subunits may be replaced with one or more foreign capsid subunits, or a portion thereof. Furthermore, the chimeric parvovirus capsid may contain insertions and/or substitutions at more than one site within the capsid. According to this embodiment, the multiple insertions/substitutions may be derived from more than one parvovirus (*e.g.*, two, three, four, five or more). Generally, it is preferred that at least one subunit from the parvovirus capsid is retained in the chimeric capsid, although this is not required.

In particular embodiments of the invention, the foreign parvovirus capsid region that is inserted or substituted into the native parvovirus capsid is at least about 2, 5, 10, 12, 15, 20, 30, 50, or 100 amino acids in length.

The inventive chimeric parvoviruses may contain any parvovirus genome, preferably an AAV genome, more preferably a recombinant AAV genome. Embodiments wherein the AAV genome is packaged within a chimeric AAV capsid of the same serotype is also preferred. AAV type 2 genomes are most preferred regardless of the composition of the chimeric parvovirus capsid.

In preferred embodiments of the invention, the chimeric parvovirus comprises an AAV capsid, more preferably an AAV type 2 capsid, in which a capsid region from a B19 parvovirus has been substituted for one of the AAV capsid domains. In other preferred embodiments, the chimeric parvovirus comprises an AAV capsid (more preferably, an AAV type 2 capsid) in which the Vp3 subunit of the AAV capsid has been replaced by the B19 Vp2 subunit.

In alternative preferred embodiments, the chimeric parvovirus comprises an AAV capsid (preferably type 2) in which the Vp1 and Vp2 subunits are replaced by the Vp1 subunit of a B19 parvovirus.

In other preferred embodiments, the chimeric parvovirus comprises an AAV type 2 capsid in which the type 2 Vp1 subunit has been replaced by the Vp1 subunit from an AAV type 1, 3, 4, 5, or 6 capsid, preferably a type 3, 4, or 5 capsid. Alternatively, the chimeric parvovirus has an AAV type 2 capsid in which the type 2 Vp2 subunit has been replaced by the Vp2 subunit from an AAV type 1, 3, 4, 5, or 6 capsid, preferably a type 3, 4, or 5 capsid. Likewise, chimeric parvoviruses in which the Vp3 subunit from an AAV type 1, 3, 4, 5 or 6 (more preferably, type 3, 4 or 5) is substituted for the Vp3 subunit of an AAV type 2 capsid are preferred. As a further alternative, chimeric parvoviruses in which two of the AAV type 2 subunits are replaced by the subunits from an AAV of a different serotype (*e.g.*, AAV type 1, 3, 4, 5 or 6) are preferred. In exemplary chimeric parvoviruses according to this embodiment, the Vp1 and Vp2, or Vp1 and Vp3, or Vp2 and Vp3 subunits of an AAV type 2 capsid are replaced by the corresponding subunits of an AAV of a different serotype (*e.g.*, AAV type 1, 3, 4, 5 or 6). Likewise, in other preferred embodiments, the chimeric parvovirus has an AAV type 1, 3, 4, 5 or 6 capsid (preferably the type 2, 3 or 5 capsid) in which one or two subunits have been replaced with those from an AAV of a different serotype, as described above for AAV type 2.

In still other preferred embodiments, the minor subunit of one parvovirus may be substituted with any minor subunit of another parvovirus (*e.g.*, Vp2 of AAV type 2 may be replaced with Vp1 from AAV type 3; Vp1 of

B19 may substitute for Vp1 and/or VP2 of AAV). Likewise, the major capsid subunit of one parvovirus may be replaced with the major capsid subunit of another parvovirus.

The nucleotide sequence of specific chimeric capsids include those encoded by the helper plasmid given in **Appendix 2** (nucleotides 2133 to 4315 of **SEQ ID NO:2**). This sequence contains the AAV2 rep coding sequences, most of the AAV2 Vp1 and Vp3 coding sequences, and the entire AAV4 Vp2 coding sequences and some of the AAV4 Vp1 and Vp3 coding sequences in a pBluescript backbone. Preferably, the chimeric parvoviruses having the capsid encoded by the helper given in **SEQ ID NO:2** carry an AAV2 genome.

Alternatively, the nucleotide sequence of the chimeric capsid is substantially homologous to the capsid coding sequence given as nucleotides 2133 to 4315 of **SEQ ID NO:2**. As a further alternative, the nucleotide squence of the chimeric capsid encodes the same amino acid sequence as nucleotides 2133 to 4315 of **SEQ ID NO:2**. The term "substantially homologous" is as defined hereinbelow.

The present invention also provides the discovery that chimeric parvoviruses may generate unique capsid structures that do not resemble the constituent parvovirus capsids. For example, the present investigations have discovered that B19/AAV type 2 chimeras, in which the Vp3 subunit of AAV type 2 has been replaced by the Vp2 subunit of a human B19 virus, results in the expected 23-28 nm particle (typical for wt AAV) and a novel 33-38 nm particle. The larger particles were present at the same density as the 23-28 nm particles in a cesium isopycnic gradient.

While not wishing to be held to any particular theory of the invention, these results suggest that this particle is formed by changing the triangulation number from T=1 to T=3, to yield a larger particle containing 180 copies of the major capsid component instead of 60. This novel particle may package larger than wild-type genomes due to its increased size. In particular preferred embodiments, the B19/AAV type 2 chimeric parvovirus capsid (B19

Vp2 swapped for AAV2 Vp3) has the sequence given as **SEQ ID NO. 3** (**Appendix 3**).

The present invention further provides B19/AAV chimeric capsids and parvoviruses having larger than wild-type capsid structures (*e.g.*, larger than about 28 nm, 30 nm, 32 nm, 34 nm, 36 nm, 38 nm, 40 nm or more in diameter). Alternatively stated, the present invention provides B19/AAV chimeric capsids and parvoviruses with capsid structures containing more than the wild-type number of capsid subunits (*e.g.*, greater than about 60 capsid subunits, greater than about 90 capsid subunits, greater than about 120 capsid subunits, greater than about 180 capsid subunits). As a further alternative statement, the present invention provides B19/AAV capsids and parvoviruses that efficiently package greater than wild-type genomes (*e.g.*, greater than about 4.8 kb, 5.0 kb, 5.2 kb, 5.4 kb, 5.6 kb, 5.8 kb, 6.0 kb, 6.2 kb, 6.4 kb, 6.6 kb, 6.8 kb or more). Preferably, the larger genomes are efficiently packaged to produce viral stocks having the titers described hereinabove.

It is also preferred that the B19/AAV chimeras have altered antigenic properties. In particular, it is preferred that the B19/AAV chimeras may be administered to a subject that has antibodies against the serotype of the AAV without immune clearance, *i.e.*, the chimera is not recognized by the AAV serotype-specific antibodies.

In other preferred embodiment of the invention, the nucleotide sequence of the B19/AAV chimeric capsid is substantially homologous to the sequence given as **SEQ ID NO:3** and encodes a chimeric parvovirus capsid. This definition is intended to include AAV of other serotypes and non-human B19 viruses. As used herein, sequences that are "substantially homologous" are at least 75%, and more preferably are 80%, 85%, 90%, 95%, or even 99% homologous or more.

High stringency hybridization conditions that permit homologous nucleotide sequences to hybridize are well known in the art. For example, hybridization of homologous nucleotide sequences to hybridize to the sequence given **SEQ ID NO:3** may be carried out in 25% formamide, 5X SSC, 5X Denhardt's solution, with 100 μg/ml of single stranded DNA and 5% dextran

sulfate at 42°C, with wash conditions of 25% formamide, 5X SSC, 0.1% SDS at 42°C for 15 minutes, to allow hybridization of sequences of about 60% homology. More stringent conditions are represented by a wash stringency of 0.3M NaCl, 0.03 M sodium citrate, 0.1% SDS at 60° or even 70° C using a standard *in situ* hybridization assay. (*See* SAMBROOK ET AL., MOLECULAR CLONING, A LABORATORY MANUAL (2d ed. 1989)).

In other preferred embodiments, the chimeric B19/AAV capsid has the amino acid sequence encoded by the sequence given in **SEQ ID NO:3** (**Appendix 4; SEQ ID NO:4**).

In other particular preferred embodiments, a non-conserved region(s) of a parvovirus capsid is inserted or substituted, preferably substituted, into another parvovirus capsid. Preferably a non-conserved region(s) is substituted for the same (*i.e.*, homologous) region from a different parvovirus. Parvovirus specific (including AAV serotype specific) characteristics are likely associated with such non-conserved regions. It is also likely that non-conserved regions can best tolerate alterations. In particular embodiments, the looped-out regions of the parvovirus major capsid subunits are swapped between two parvoviruses, more preferably an AAV and a parvovirus, still more preferably between two AAV of different serotypes.

With particular respect to AAV type 2, although the crystal structure of this virus has not been solved, structural correlations have been made based on sequence information. The structural correlations suggest that the Vp3 subunit of AAV type 2 has eight β-barrel motifs, and that these motifs are separated by looped out regions (Chapman *et al.*, *Virology* **194**:419). Recently, the sequence of AAV type 3 has been determined by Muramatsu *et al.*, (1996) *Virology* **221**:208. The amino acid homology between Vp3 of AAV type 2 and AAV type 3 is 89%, with the region defined as loop 3/4 having 70% homology (*Id.*). Additionally, AAV type 3 does not bind to the same receptor as AAV type 2 (Mizukami *et al.*, *Virology* **217**:124). The divergent amino acid sequences in loops 3 and 4 may explain the differences in cellular receptors used by AAV type 2 and AAV type 3, and the resulting disparities in cellular tropism. Accordingly, in preferred embodiments of the instant

invention, chimeric AAV particles are constructed in which loop 3/4, or a portion thereof, of AAV type 2 is swapped for the AAV type 3 loop 3/4, or vice versa.

In other embodiments, the chimeric parvovirus comprises an AAV type 2 capsid in which loop 1, 2, 3, and/or 4 of the Vp3 subunit have been replaced by the corresponding loop region(s) of an AAV of a different serotype (*e.g.*, type 1, 3, 4, 5 or 6). In illustrative embodiments, the loop 2-4 region of the AAV type 2 Vp3 subunit is replaced by the loop 2-4 region of a type 3 or type 4 virus.

Likewise, in other preferred embodiments, the chimeric parvovirus comprises an AAV type 1, 3, 4, 5 or 6 capsid in which the loop 1, 2, 3 and/or 4 region of the Vp3 subunit is replaced by the corresponding region of a different AAV serotype. Exemplary embodiments include, but are not limited to, a chimeric parvovirus comprising an AAV type 3 or type 4 capsid in which the loop 2-4 region of the Vp3 subunit is replaced by the AAV type 2 loop 2-4 region.

The present invention further provides chimeric parvoviruses comprising an AAV capsid in which a loop region(s) in the major Vp3 subunit is replaced by a loop region (s) (preferably, a corresponding loop region(s)) from the major subunit of an autonomous parvovirus. In particular, the loop region 1, 2, 3 and/or 4 from an AAV type 1, 2, 3, 4, 5, or 6 Vp3 subunit is replaced with a loop region from the major subunit of an autonomous parvovirus.

The nucleotide sequence of specific chimeric capsids include those having the capsid sequence encoded by the helper plasmid given in **Appendix 5** (nucleotides 2133 to 4342 of **SEQ ID NO:5**). This sequence contains the AAV2 rep coding sequences, most of the AAV2 capsid coding sequences, with the exception that loops 2-4 from the AAV2 Vp3 subunit were replaced with the corresponding region from AAV3, in a pBluescript backbone.

Alternatively, the nucleotide sequence of the chimeric capsid is substantially homologous to the sequence given as nucleotides 2133 to 4342 of

SEQ ID NO:5. As a further alternative, the nucleotide sequence of the chimeric capsid has the same amino acid sequence as the capsid encoded by nucleotides 2133 to 4342 of **SEQ ID NO:5**. The term "substantially homologous" is as defined hereinabove.

Chimeric parvoviruses may be constructed as taught herein or by other standard methods known in the art. Likewise, those skilled in the art may evaluate the chimeric parvoviruses thus generated for assembly, packaging, cellular tropism, and the like, as described herein or by other standard methods known in the art, without undue experimentation.

Another aspect of the present invention is a chimeric parvovirus capsid protein (preferably an AAV Vp1, Vp2 or Vp3 capsid protein) with at least one capsid region from another parvovirus(es) inserted or substituted therein (preferably, substituted). The introduction of the foreign capsid protein into a parvovirus capsid provides altered characteristics (*e.g.*, immunogenic, tropism, *etc.*) to a virus capsid or particle (preferably a parvovirus capsid or particle) incorporating the chimeric parvovirus capsid protein. Alternatively, the chimeric parvovirus capsid protein may facilitate detection or purification of a virus capsid or particle (preferably parvovirus capsid or particle) incorporating the chimeric parvovirus capsid protein. In particular preferred embodiments, the antigenic properties of an AAV capsid or particle of a particular serotype may be altered (*e.g.*, changed or modified) or diminished (*e.g.*, reduced or mitigated) by incorporation of the chimeric parvovirus capsid region for the native capsid region. According to this embodiment, chimeric capsid proteins may be used to obviate or reduce immune clearance in subjects that have immunity against the serotype of the AAV capsid or particle (*e.g.*, to permit multiple virus administrations). Changes or reductions in antigenic properties may be assessed, *e.g.*, in comparison to an AAV capsid or particle that is identical except for the presence of the chimeric parvovirus capsid protein.

The present invention also encompasses empty chimeric parvovirus capsid structures. Empty capsids may be used for presentation or delivery of peptides or proteins (*e.g.*, antigens to produce an immune response), nucleic

acids, or other compounds (*see, e.g.*, Miyamura *et al.*, (1994) *Proc. Nat. Acad. Sci USA* **91**:8507; U.S. Patent No. 5,916,563 to Young *et al.*, 5,905,040 to Mazzara *et al.*, U.S. Patent No. 5,882,652, U.S. Patent No. 5,863,541 to Samulski *et al.*; the disclosures of which are incorporated herein in their entirety by reference). Empty capsids may be produced by any method known in the art. (*see, e.g., id.*).

The chimeric parvoviruses and capsids of the invention further find use in raising antibodies against the novel capsid structures. Antibodies may be produced by methods that are known to those skilled in the art.

The present invention also provides cloning vectors, transcomplementing packaging vectors, packaging cells, and methods for producing the inventive chimeric parvovirus particles disclosed herein. In general, vectors, packaging cells, and methods for producing chimeric parvoviruses are as described above with respect to hybrid parvoviruses. In addition, at least one of the cap genes (encoded by the rAAV genome, a packaging vector(s), or the packaging cell) has inserted therein at least one nucleic acid sequence encoding a foreign amino acid sequence from a non-homologous parvovirus (as described above).

III. Targeted Parvoviruses.

A further aspect of the present invention are parvovirus vectors comprising a parvovirus capsid and a recombinant AAV genome, wherein an exogenous targeting sequence has been inserted or substituted into the parvovirus capsid. The parvovirus vector is preferably targeted (*i.e.*, directed to a particular cell type or types) by the substitution or insertion of the exogenous targeting sequence into the parvovirus capsid. Alternatively stated, the exogenous targeting sequence preferably confers an altered tropism upon the parvovirus. As yet a further alternative statement, the targeting sequence increases the efficiency of delivery of the targeted vector to a cell.

As is described in more detail below, the exogenous targeting sequence may be a virus capsid sequence (*e.g.*, an autonomous parvovirus

capsid sequence, AAV capsid sequence, or any other viral capsid sequence) that directs infection of the parvovirus to a particular cell type(s). As an alternative, the exogenous amino acid sequence may encode any peptide or protein that directs entry of the parvovirus vectors into a cell(s). In preferred embodiments, the parvovirus capsid is an AAV capsid, more preferably, an AAV type 2 capsid.

An "altered" tropism, as used herein, includes reductions or enhancements in infectivity with respect to a particular cell type(s) as compared with the native parvovirus lacking the targeting sequence(s). An "altered" tropism also encompasses the creation of a new tropism (*i.e.*, the parvovirus would not infect a particular cell type(s) to a significant or, alternatively, a detectable extent in the absence of the exogenous amino acid sequence). Alternatively, an "altered tropism" may refer to a more directed targeting of the parvovirus vector to a particular cell type(s) as compared with the native parvovirus, but the target cells may typically be infected by the native parvovirus as well (*e.g.*, a narrowed tropism). As a further alternative, an "altered" tropism refers to a more efficient delivery of a targeted parvovirus as compared with the native parvovirus (*e.g.*, a reduced Multiplicity of Infection, "MOI").

The term "reduction in infectivity", as used herein, is intended to encompass both an abolishment of the wild-type tropism as well as a diminishment in the wild-type tropism or infectivity toward a particular cell type(s). The diminished infectivity may be a 25%, 50%, 75%, 90%, 95%, 99%, or more decrease in infectivity with respect to the wild-type level of infectivity. By "enhancement in infectivity", it is meant that the infectivity with respect to a particular cell type(s) is increased above that observed with the wild-type parvovirus, *e.g.*, by at least 25%, 50%, 75%, 100%, 150%, 200%, 300%, or 500%, or more.

The exogenous targeting sequence(s) may replace or substitute part or all of a capsid subunit, alternatively, more than one capsid subunit. As a further alternative, more than one exogenous targeting sequence (*e.g.*, two, three, four, five or more sequences) may be introduced into the parvovirus

capsid. In alternative embodiments, insertions and substitutions within the minor capsid subunits (*e.g.*, Vp1 and Vp2 of AAV) are preferred. For AAV capsids, insertions or substitutions in Vp2 or Vp3 are also preferred.

Those skilled in the art will appreciate that due to the overlap in the sequences encoding the parvovirus capsid proteins, a single insertion or substitution may affect more than one capsid subunit.

As described above, in particular embodiments, the present invention provides chimeric parvovirus particles with unique structures and properties. The substitution and/or insertion of one or more parvovirus capsid region(s) for another to create a chimeric parvovirus capsid may result in the loss of the wild-type parvovirus tropism and/or the development of a new tropism associated with the exogenous capsid region(s). Accordingly, targeted parvoviruses may also be chimeric parvoviruses as is described in more detail hereinabove. In particular, targeted chimeric parvoviruses are provided in which a capsid subunit(s) or a loop region(s) from the major capsid subunit has been replaced with a capsid subunit(s) or loop region from another parvovirus.

Accordingly, in particular embodiments of the instant invention, chimeric parvovirus particles are constructed in which the capsid domains that encode the wild-type parvovirus tropism are swapped with capsid regions or subunits from a different parvovirus sequence, thereby diminishing or even completely abolishing the wild-type tropism. These infection-negative parvoviruses find use as templates for creating parvoviruses with targeted tropisms. In this manner, a parvovirus with a new or directed tropism, but lacking the wild-type tropism, may be generated.

In another preferred embodiment, a parvovirus capsid region that directs the native or wild-type tropism is swapped with a capsid domain that directs the tropism of another parvovirus, thereby diminishing or ablating the native tropism and concurrently conferring a new tropism to the chimeric parvovirus. In other embodiments, the foreign capsid region is substituted or inserted into the parvovirus capsid without reducing or extinguishing the wild-type tropism. As a further alternative, more than one foreign parvovirus

capsid region (*e.g.*, two, three, four, five, or more) is swapped into the parvovirus capsid. For example, a first foreign capsid region may replace the native capsid region directing the wild-type tropism. Additional foreign capsid regions provide the chimeric capsid with a new tropism(s).

Heparan sulfate (HS) has recently been identified as a primary receptor for AAV (Summerford and Samulski, (1998) *J. Virology* **72**:1438). Thus, the capsid structure may be modified to facilitate or enhance binding of AAV to the cellular receptor or to inhibit or prevent binding thereto. To illustrate, the tropism of the AAV may be altered by swapping out the HS binding domain for the AAV capsid, for example, with sequences from other parvoviruses that do contain this HS binding domain or any other sequences.

Several consensus sequences have been identified among ligands that bind to HS receptors. In general, HS appears to bind to sequences including clusters of basic amino acids. Illustrative consensus sequences include but are not limited to BBXB, BBBXXB, and $RX_7FRXKKXXXK$, where B is a basic amino acid, and X is any amino acid. Three sequences containing clusters of basic amino acids are present in the first 170 amino acid residues of the VP1 capsid protein of AAV type 2 as follows: RX_5KKR at amino acids 116 to 124, KX_4KKR at amino acids 137 to 144, and KX_6RKR at amino acids 161 to 170 (AAV type 2 sequence and numbering as described by Srivastava *et al.*, (1983) *J. Virology* **45**:555, as modified by Ruffing *et al.*, (1994) *J. Gen. Virology* **75**:3385, Muzyczka, (1992) *Curr. Topics Microbiol. Immunol.* **158**:97, and Cassinotti *et al.*, (1988) *Virology* **167**:176). In addition, the consensus sequence ($RX_7FRPKRLNFK$) is found in the VP1 capsid subunit of AAV type 2 at amino acids 299 to 315.

It appears that AAV serotypes 4 and 5 do not bind to cellular HS receptors, or do so with a low efficiency. Accordingly, in particular embodiments, the HS binding domain of AAV serotypes 1, 2, 3, or 6 may be replaced with the corresponding region of AAV serotype 4 or 5 to reduce or abolish HS binding. Likewise, HS binding may be conferred upon AAV serotype 4 or 5 by inserting or substituting in the HS binding domain from AAV 1, 2, 3 or 6.

The HS consensus sequences are marked by an abundance of basic amino acids. There is a high density of positively charged amino acids within the first 170 residues of the AAV type 2 Vp1 Cap protein, including three strings of basic amino acids, which may be involved in an ionic interaction with the cell surface. Accordingly, in one particular embodiment of the invention, the affinity of an AAV capsid for HS receptors is reduced or eliminated by creating a targeted parvovirus in which some or all of the basic sequences are substituted by other sequences, *e.g.*, from another parvovirus that does not contain the HS binding domain.

Alternatively, the respiratory syncytial virus heparin binding domain may be inserted or substituted into a virus that does not typically bind HS receptors (*e.g.*, AAV 4, AAV5, B19) to confer heparin binding to the resulting mutant.

B19 infects primary erythroid progenitor cells using globoside as its receptor (Brown *et al.*, (1993) Science **262**:114). The structure of B19 has been determined to 8 Å resolution (Agbandje-McKenna *et al.*, (1994) *Virology* **203**:106). The region of the B19 capsid that binds to globoside has been mapped between amino acids 399-406 (Chapman *et al.*, (1993) *Virology* **194**:419), a looped out region between β-barrel structures E and F (Chipman *et al.*, (1996) *Proc. Nat. Acad. Sci. USA* **93**:7502). Accordingly, the globoside receptor binding domain of the B19 capsid may be inserted/substituted into other parvovirus capsids (preferably an AAV capsid, more preferably, the AAV type 2 capsid) to target the resulting chimeric parvovirus to erythroid cells.

In more preferred embodiments, the exogenous targeting sequence may be any amino acid sequence encoding a peptide or protein, which is inserted or substituted into the parvovirus capsid to alter the tropism of the parvovirus. The native parvovirus tropism may be reduced or abolished by insertion or substitution of the amino acid sequence. Alternatively, the insertion or substitution of the exogenous amino acid sequence may target the parvovirus to a particular cell type(s). In yet further preferred embodiments, an exogenous targeting sequence is substituted or inserted

into the parvovirus capsid to concurrently ablate the wild type tropism and to introduce a new tropism. For example, a targeting peptide may be inserted directly into a targeting region of the AAV capsid to simultaneously disrupt the native tropism (*e.g.*, by interfering with binding to cellular heparan sulfate receptors) and to direct the targeted AAV vector to particular cells.

Those skilled in the art will appreciate that the native tropism of a parvovirus may be reduced or abolished without substituting or inserting an exogenous targeting sequence directly into those regions of the parvovirus capsid responsible for the receptor binding. Mutants that have lost the wild-type tropism are useful as templates for the creation of parvoviruses with novel tropisms as taught herein. It is preferred that substitutions or insertions that result in the loss of wild-type tropism act at the level of receptor binding and/or entry into the cell. In other words, it is preferred that the altered parvovirus is otherwise capable of infecting a cell if entry into the cell is provided by other means, *e.g.*, by a bispecific antibody, by targeting peptide or protein as disclosed herein, or by any other means known in the art.

The exogenous targeting sequence may be any amino acid sequence encoding a protein or peptide that alters the tropism of the parvovirus. In particular embodiments, the targeting peptide or protein may be naturally occurring or, alternately, completely or partially synthetic. Exemplary peptides and proteins include ligands and other peptides that bind to cell surface receptors and glycoproteins, such as RGD peptide sequences, bradykinin, hormones, peptide growth factors (*e.g.*, epidermal growth factor, nerve growth factor, fibroblast growth factor, platelet-derived growth factor, insulin-like growth factors I and II, *etc.*), cytokines, melanocyte stimulating hormone (*e.g.*, α, β or γ), neuropeptides and endorphins, and the like, and fragments thereof that retain the ability to target cells to their cognate receptors. Other illustrative peptides and proteins include substance P, keratinocyte growth factor, neuropeptide Y, gastrin releasing peptide, interleukin 2, hen egg white lysozyme, erythropoietin, gonadoliberin, corticostatin, β-endorphin, leu-enkephalin, rimorphin, α-neo-enkephalin, angiotensin, pneumadin, vasoactive intestinal peptide, neurotensin, motilin, and fragments thereof as described

above. As a further alternative, the targeting peptide or protein may be an antibody or Fab fragment that recognizes, *e.g.*, a cell-surface epitope, such as an anti-receptor antibody. As yet a further alternative, the binding domain from a toxin (*e.g.*, tetanus toxin or snake toxins, such as α-bungarotoxin, and the like) can be used to target the inventive parvovirus vectors to particular target cells of interest. In a yet further preferred embodiment the parvovirus vectors may be delivered to a cell using a "nonclassical" import/export signal peptide (*e.g.*, fibroblast growth factor-1 and –2, interleukin 1, HIV-1 Tat protein, herpes virus VP22 protein, and the like) as described by Cleves, (1997) *Current Biology* **7**:R318. Also encompassed are peptide motifs that direct uptake by specific cells, *e.g.*, a FVFLP peptide motif triggers uptake by liver cells. Phage display techniques, as well as other techniques known in the art, may be used to identify peptides that recognize, preferably specifically, any cell type of interest.

The term "antibody" as used herein refers to all types of immunoglobulins, including IgG, IgM, IgA, IgD, and IgE. The antibodies may be monoclonal or polyclonal and may be of any species of origin, including (for example) mouse, rat, rabbit, horse, or human, or may be chimeric antibodies. Also encompassed by the term "antibody" are bispecific or "bridging" antibodies as known by those skilled in the art.

Antibody fragments within the scope of the present invention include, for example, Fab, F(ab')2, and Fc fragments, and the corresponding fragments obtained from antibodies other than IgG. Such fragments may be produced by known techniques.

The targeting sequence may alternatively encode any peptide or protein that targets the parvovirus particle to a cell surface binding site, including receptors (*e.g.*, protein, carbohydrate, glycoprotein or proteoglycan), as well as any oppositely charged molecule (as compared with the targeting sequence or the parvovirus capsid), or other molecule with which the targeting sequence or targeted parvovirus interact to bind to the cell, and thereby promote cell entry. Examples of cell surface binding sites include, but are not limited to, heparan sulfate, chondroitin sulfate, and other

glycosaminoglycans, sialic acid moieties found on mucins, glycoproteins, and gangliosides, MHC I glycoproteins, carbohydrate components found on membrane glycoproteins, including, mannose, N-acetyl-galactosamine, N-acetyl-glucosamine, fucose, galactose, and the like.

As yet a further alternative, the targeting sequence may be a peptide or protein that may be used for chemical coupling (*e.g.*, through amino acid side groups of arginine or lysine residues) to another molecule that directs entry of the parvovirus into a cell.

In other embodiments, the exogenous targeting sequence is substituted or inserted into the capsid to disrupt binding to cellular receptors (*e.g.*, HS receptor) and/or entry into the cell. For example, the exogenous amino acid sequence may be substituted or inserted into the region(s) of the AAV capsid that binds to cellular receptors and/or otherwise mediates entry of the virus into the cell. Preferably, the exogenous targeting sequence is inserted into the capsid region(s) that interact with cellular HS receptors (as described above). One illustrative insertion mutant that forms intact AAV virions yet fails to bind heparin agarose or infect Hela cells is an AAV type 2 mutant generated by insertion of an amino acid sequence at bp 3761 of the AAV type 2 genome (within the Vp3 cap gene region).

In a further alternative embodiment, the exogenous amino acid sequence inserted into the parvovirus capsid may be one that facilitates purification of the parvovirus. According to this aspect of the invention, it is not necessary that the exogenous amino acid sequence also alters the tropism of the modified parvovirus. For example, the exogenous amino acid sequence may include a poly-histidine sequence that is useful for purifying the parvovirus over a nickel column, as is known to those skilled in the art. Alternatively, the region of the AAV capsid that interacts with heparin and/or heparan sulfate may be substituted or inserted into a parvovirus capsid so that the parvovirus may be purified by binding to heparin, *e.g.*, as described by Zolotukhin *et al.*, (1999) *Gene Therapy* **6**:973, the disclosure of which is incorporated herein in its entirety by reference.

In other embodiments, the amino acid sequence encodes an antigenic peptide or protein that may be employed to purify the AAV by standard immunopurification techniques. Alternatively, the amino acid sequence may encode a receptor ligand or any other peptide or protein that may be used to purify the modified parvovirus by affinity purification or any other techniques known in the art (*e.g.*, purification techniques based on differential size, density, charge, or isoelectric point, ion-exchange chromatography, or peptide chromatography).

In yet other embodiments of the invention, an amino acid sequence may be inserted or substituted into a parvovirus particle to facilitate detection thereof (*e.g.*, with a antibody or any other detection reagent, as is known in the art). For example, the "flag" epitope may be inserted into the parvovirus capsid and detected using commercially-available antibodies (Eastman-Kodak, Rochester, NY). Detectable viruses find use, *e.g.*, for tracing the presence and/or persistence of virus in a cell, tissue or subject.

In still a further embodiment, an exogenous amino acid sequence encoding any antigenic protein may be expressed in the modified capsid (*e.g.*, for use in a vaccine).

As described below and in **Table I**, the present investigations have used insertional mutagenesis of the capsid coding sequence of AAV serotype 2 in order to determine positions within the capsid that tolerate peptide insertions. Viable mutants were identified with insertions throughout each of the capsid subunits. These insertion mutants find use for any purpose in which it is desirable to insert a peptide or protein sequence into an AAV capsid, *e.g.*, for purifying and/or detecting virus, or for inserting an antigenic peptide or protein into the capsid. The nucleotide positions indicated in **Table 1** (see Examples) are the positions at which the restriction sites were made, *e.g.*, the new sequences start at the next nucleotide. For example, for an insertion mutant indicated in **Table 1** as having an insertion at nucleotide 2285, the new insertion sequence would begin at nucleotide 2286.

It is preferred to insert the exogenous amino acid sequence within the parvovirus minor Cap subunits, *e.g.*, within the AAV Vp1 and Vp2 subunits.

Alternately, insertions in Vp2 or Vp3 are preferred. Also preferred are insertion mutations at nucleotide 2285, 2356, 2364, 2416, 2591, 2634, 2690, 2747, 2944, 3317, 3391, 3561, 3595, 3761, 4046, 4047, and/or 4160 within the AAV type 2 cap genes, preferably, to generate an AAV type 2 vector with an altered tropism as described herein (AAV type 2 numbering used herein is as described by Srivastava *et al.*, (1983) *J. Virology* **45**:555, as modified by Ruffing *et al.*, (1994) *J. Gen. Virology* **75**:3385, Muzyczka, (1992) *Curr. Topics Microbiol. Immunol.* **158**:97, and Cassinotti *et al.*, (1988) *Virology* **167**:176).

Insertions at these nucleotide positions for AAV2 will give rise to amino acid insertions following amino acid 28 (nu 2285), 51 (nu 2356), 54 (nu 2364), 71 (nu 2416), 130 (nu 2591), 144 (nu 2634), 163 (nu 2690), 182 (nu 2747), 247 (nu 2944), 372 (nu 3317), 396 (nu 3391), 452 (nu 3561), 464 (nu 3595), 520 (nu 3761), 521 (nu 3766), 615 (nu 4046 and 4047), and 653 (nu 4160) within the AAV2 capsid coding region (using the starting methionine residue for Vp1 as amino acid 1), or the corresponding regions of AAV of other serotypes as known by those skilled in the art. Those skilled in the art will appreciate that due to the overlap in the AAV capsid coding regions, these insertions may give rise to insertions within more than one of the capsid proteins (**Table 2**).

Table 2

Insertion Positions in AAV2 Capsid[1,2]

Insertion site (nucleotide)	Vp1 (amino acid)	Vp2 (amino acid)	Vp3 (amino acid)
2285	28	--	--
2356	51	--	--
2364	54	--	--
2416	71	--	--
2591	130	--	--
2634	144	7	--
2690	163	26	--

2747	182	45	--
2944	247	110	45
3317	372	235	170
3391	396	259	194
3561	452	315	250
3595	464	327	262
3753	517	380	315
3761	520	383	318
3766	521	384	319
3789	529	392	327
3858	552	415	350
3960	586	449	384
3961	586	449	384
3987	595	458	393
4046	615	478	413
4047	615	478	413
4160	653	516	451

[1]The indicated nucleotide or amino acid refers to the nucleotide or amino acid immediately preceding the inserted sequence.

[2]Vp1 start at nucleotide 2203

Alternatively, the exogenous amino acid sequence is inserted at the homologous sites to those described above in AAV capsids of other serotypes as known by those skilled in the art (*see*, *e.g.*, Chiorini *et al.*, (1999) *J. Virology* **73**:1309). The amino acid positions within the AAV capsid appear to be highly, or even completely, conserved among AAV serotypes. Accordingly, in particular embodiments, the exogenous amino acid sequence is substituted at the amino acid positions indicated in **Table 2** (new sequence starting at the next amino acid) in AAV other than serotype 2 (*e.g.*, serotype 1, 3, 4, 5 or 6).

As further alternatives, an exogenous amino acid sequence may be inserted into the AAV capsid at the positions described above to facilitate purification and/or detection of the modified parvovirus or for the purposes of antigen presentation, as described above.

One particular AAV type 2 mutant is produced by inserting an amino acid sequence at nucleotide position 2634 of the genome (within the Vp2 *cap* gene region; AAV2 numbering as described above). This mutant forms AAV type 2 virions with normal morphology by electron microscopy analysis in the absence of detectable expression of the Vp1 and Vp2 subunits. Moreover, this mutant protects the viral genome and retains binding to a heparin-agarose matrix, although it does not demonstrate infectivity in HeLa cells. This mutant is useful for administration to subjects to avoid an immune response against the Vp1 and Vp2 subunits. It further finds use for insertion of large peptides or proteins into the AAV capsid structure. As one illustrative example, the adenovirus knob protein is inserted into this mutant to target the virus to the Coxsackie adenovirus receptor (CAR).

Another particular AAV type 2 insertion mutant is produced by insertion of an exogenous amino acid sequence at bp 3761 of the genome (within the Vp3 capsid coding region). This mutant protects the viral genome and forms morphologically normal capsid structures, but does not bind heparin-agarose and fails to infect HeLa cells. This mutant is particularly useful as a reagent for creating AAV vectors lacking the native tropism. For example, a new targeting region may be introduced into this mutant at bp 3761 or at another site. As shown in **Table 1**, the present investigations have discovered a variety of positions within the AAV capsid that tolerate insertion of exogenous peptides and retain infectivity (*e.g.*, at bp 2356, 2591, 2690, 2944, 3595, and/or 4160 of the AAV type 2 genome).

In other preferred embodiments, AAV vectors with multiple insertions and/or substitutions are created to provide AAV vectors exhibiting a desired pattern of infectivity, *e.g.*, a non-infectious insertion/substitution mutation and an infectious mutation (*e.g.*, as shown in **Table 1**) may be combined in a single AAV vector. As one illustrative example, a peptide insertion may be

made at bp 3761 of the AAV type 2 genome (within the Vp3 subunit) to create a non-infectious heparin binding negative mutant. A second peptide insertion may be made at bp 2356 (alternatively, bp 2591, 2690, 2944, 3595 or 4160) to target the vector. The inserted peptide may be one that directs the AAV type 2 vector to target cells of interests. In particular embodiments, bradykinin may be inserted at any of the foregoing sites to target the vector to lung epithelial cells (*e.g.*, for the treatment of cystic fibrosis or other lung disorders) or the adenovirus knob protein may be inserted at the foregoing sites to target the vector to cells expressing CAR receptors. Alternatively, this vector may be employed for antigen presentation to produce an immune response.

In other embodiments, the substitution or insertion (preferably insertion) is made at nucleotides 3789 or 3961 of the AAV2 genome (*e.g.*, new sequence would start at nu 3790 and 3962, respectively), or the corresponding site of other AAV serotypes as known by those skilled in the art. These positions correspond to insertions following amino acid 529 and 586, respectively, of the AAV2 capsid (Met #1 of Vp1 as amino acid 1; **Table 2**). In particular embodiments, there will be missense mutation at nucleotides 3790-3792 (Glu → Ile) or at nucleotides 3960-3961 (Gly → Val), respectively, due to the creation of a restriction site as part of the cloning strategy. In preferred embodiments of the invention, a targeting insertion at nu 3789 or 3961 is combined with the 3761 mutation, which results in loss of heparin binding, to create a targeted capsid or parvovirus.

In other preferred embodiments an insertion or substitution (preferably, insertion) is made in the AAV2 capsid at nucleotides 3753, 3858, 3960, or 3987 (new sequence beginning at the next nucleotide), or the corresponding sites in AAV of other serotypes. These sites correspond to insertions or substitutions following amino acids 517, 552, 586, or 595, respectively, of the AAV2 capsid (Met #1 of Vp1 as amino acid 1; **Table 2**), or the corresponding sites in AAV capsids of other serotypes as known by those skilled in the art.

In other preferred embodiments, the insertion or substitution is made following amino acid 517, 529, 552, 586 or 595 of AAV capsids of other serotypes, *e.g.* (1, 2, 3, 5 or 6).

There is no particular lower or upper limit to the length of the amino acid sequence that may be inserted or substituted into the virus capsid, as long as the targeted or modified parvovirus capsid retains the desired properties (*e.g.*, assembly, packaging, infectivity). The exogenous amino acid sequence may be as short as 100, 50, 20, 16, 12, 8, 4 or 2 amino acids in length. Similarly, the exogenous amino acid sequence to be inserted/substituted into the parvovirus capsid may be as long as 2, 5, 10, 12, 15, 20, 50, 100, 200, 300 or more amino acids. In particular embodiments, the exogenous amino acid sequence encodes an entire protein. Preferably, the exogenous amino acid sequence that is inserted/substituted into the parvovirus capsid is expressed on the outside surface of the modified parvovirus capsid.

The present invention further provides targeted parvovirus capsid proteins, whereby a targeting sequence(s) is inserted or substituted into a parvovirus capsid protein, as described above. The targeted parvovirus capsid protein confers an altered tropism upon a virus vector or virus capsid (preferably, a parvovirus vector or capsid) incorporating the targeted parvovirus capsid protein therein as compared with the tropism of the native virus vector or virus capsid in the absence of the targeted parvovirus capsid protein. Likewise, modified capsid proteins (modifications as described above for parvoviruses) are another aspect of the invention. The modified capsid protein may be incorporated into a parvovirus capsid or particle, *e.g.*, to facilitate purification and/or detection thereof or for the purposes of antigen presentation.

Further provided are targeted and/or modified parvovirus capsids as described in more detail above in connection with chimeric parvovirus capsids. In particular embodiments, the present invention provides targeted parvovirus "capsid vehicles", as has been described for AAV capsids, *e.g.*, U.S. Patent No. 5,863,541.

Molecules that may be packaged by the inventive parvovirus capsids and transferred into a cell include recombinant AAV genomes, which may advantageously may then integrate into the target cell genome, and other heterologous DNA molecules. RNA, proteins and peptides, or small organic molecules, or combinations of the same. Heterologous molecules are defined as those that are not naturally found in an parvovirus infection, *i.e.*, those not encoded by the parvovirus genome. In a preferred embodiment of the present invention, a DNA sequence to be encapsidated may be linked to an AAV ITR sequence that contains the viral packaging signals, which may increase the efficiency of encapsidation and/or targeted integration into the genome.

The invention is further directed to the association of therapeutically useful molecules with the outside of the inventive parvovirus capsids for transfer of the molecules into host target cells. Such associated molecules may include DNA, RNA, carbohydrates, lipids, proteins or peptides. In one embodiment of the invention the therapeutically useful molecules is covalently linked (*i.e.*, conjugated or chemically coupled) to the capsid proteins. Methods of covalently linking molecules are known by those skilled in the art.

The targeted and/or modified parvovirus capsid proteins, capsids, and virus particles of the invention find use for raising antibodies against these novel capsid structures. Alternatively, an exogenous amino acid sequence may be inserted into the parvovirus capsid for antigen presentation to a cell, *e.g.* for administration to a subject to produce an immune response to the exogenous amino acid sequence. According to this latter embodiment, it is not necessary that the exogenous amino acid sequence also alter the tropism of the parvovirus.

It will be appreciated by those skilled in the art that modified/targeted viruses and capsids as described above may also be chimeric and/or hybrid parvoviruses as described in the preceding sections. Those skilled in the art will further appreciate that the insertion mutants described herein include parvoviruses with other modifications, *e.g.*, deletion, insertion or missense

mutations. In addition, the mutations may incidentally be introduced into the parvovirus capsid or rAAV genome as a result of the particular cloning strategy employed.

Parvoviruses, AAV, and rAAV genomes are as described above with respect to hybrid parvoviruses. The present invention also provides cloning vectors, transcomplementing packaging vectors, packaging cells, and methods for producing the modified and/or targeted rAAV particles described above. In general, helpers, packaging cells, and methods for producing the targeted or modified parvoviruses are as described above with respect to hybrid and chimeric viruses. In addition, at least one of the cap genes (encoded by the rAAV genome, a packaging vector, or the packaging cell) has inserted or substituted therein at least one nucleic acid sequence encoding an exogenous targeting sequence (as described above) or an exogenous amino acid sequence (as described above, *e.g.*, for purification, detection or antigen presentation).

IV. Gene Transfer Technology.

The methods of the present invention provide a means for delivering heterologous nucleic acid sequences into a broad range of host cells, including both dividing and non-dividing cells. The vectors and other reagents, methods and pharmaceutical formulations of the present invention are additionally useful in a method of administering a protein or peptide to a subject in need thereof, as a method of treatment or otherwise. In this manner, the protein or peptide may thus be produced *in vivo* in the subject. The subject may be in need of the protein or peptide because the subject has a deficiency of the protein or peptide, or because the production of the protein or peptide in the subject may impart some therapeutic effect, as a method of treatment or otherwise, and as explained further below.

In general, the present invention may be employed to deliver any foreign nucleic acid with a biological effect to treat or ameliorate the symptoms associated with any disorder related to gene expression. Illustrative disease states include, but are not limited to: cystic fibrosis (and

other diseases of the lung), hemophilia A, hemophilia B, thalassemia, anemia and other blood disorders, AIDs, Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, epilepsy, and other neurological disorders, cancer, diabetes mellitus, muscular dystrophies (*e.g.*, Duchenne, Becker), Gaucher's disease, Hurler's disease, adenosine deaminase deficiency, glycogen storage diseases and other metabolic defects, retinal degenerative diseases (and other diseases of the eye), diseases of solid organs (*e.g.*, brain, liver, kidney, heart), and the like.

Gene transfer has substantial potential use in understanding and providing therapy for disease states. There are a number of inherited diseases in which defective genes are known and have been cloned. In some cases, the function of these cloned genes is known. In general, the above disease states fall into two classes: deficiency states, usually of enzymes, which are generally inherited in a recessive manner, and unbalanced states, at least sometimes involving regulatory or structural proteins, which are inherited in a dominant manner. For deficiency state diseases, gene transfer could be used to bring a normal gene into affected tissues for replacement therapy, as well as to create animal models for the disease using antisense mutations. For unbalanced disease states, gene transfer could be used to create a disease state in a model system, which could then be used in efforts to counteract the disease state. Thus the methods of the present invention permit the treatment of genetic diseases. As used herein, a disease state is treated by partially or wholly remedying the deficiency or imbalance that causes the disease or makes it more severe. The use of site-specific integration of nucleic sequences to cause mutations or to correct defects is also possible.

The instant invention may also be employed to provide an antisense nucleic acid to a cell *in vitro* or *in vivo*. Expression of the antisense nucleic acid in the target cell diminishes expression of a particular protein by the cell. Accordingly, antisense nucleic acids may be administered to decrease expression of a particular protein in a subject in need thereof. Antisense nucleic acids may also be administered to cells *in vitro* to regulate cell

physiology, *e.g.*, to optimize cell or tissue culture systems. The present invention is also useful to deliver other non-translated RNAs, *e.g.*, ribozymes (*e.g.*, as described in U.S. Patent No. 5,877,022), RNAs that effect spliceosome-mediated *trans*-splicing (Puttaraju *et al.*, (1999) *Nature Biotech.* **17**:246), or "guide" RNAs (*see, e.g.*, Gorman *et al.*, (1998) *Proc. Nat. Acad. Sci. USA* **95**:4929; U.S. Patent No. 5,869,248 to Yuan *et al.*) to a target cell.

Finally, the instant invention finds further use in diagnostic and screening methods, whereby a gene of interest is transiently or stably expressed in a cell culture system, or alternatively, a transgenic animal model.

V. Subjects, Pharmaceutical Formulations, Vaccines, and Modes of Administration.

The present invention finds use in both veterinary and medical applications. Suitable subjects include both avians and mammals, with mammals being preferred. The term "avian" as used herein includes, but is not limited to, chickens, ducks, geese, quail, turkeys and pheasants. The term "mammal" as used herein includes, but is not limited to, humans, bovines, ovines, caprines, equines, felines, canines, lagomorphs, *etc.* Human subjects are the most preferred. Human subjects include fetal, neonatal, infant, juvenile and adult subjects.

In particular embodiments, the present invention provides a pharmaceutical composition comprising a virus particle of the invention in a pharmaceutically-acceptable carrier or other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, *etc.* For injection, the carrier will typically be a liquid. For other methods of administration, the carrier may be either solid or liquid, such as sterile, pyrogen-free water or sterile pyrogen-free phosphate-buffered saline solution. For inhalation administration, the carrier will be respirable, and will preferably be in solid or liquid particulate form. As an injection medium, it is preferred to use water that contains the additives usual for injection solutions, such as stabilizing agents, salts or saline, and/or buffers.

In other embodiments, the present invention provides a pharmaceutical composition comprising a cell in which an AAV provirus is integrated into the genome in a pharmaceutically-acceptable carrier or other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, *etc.*

By "pharmaceutically acceptable" it is meant a material that is not biologically or otherwise undesirable, *e.g.*, the material may be administered to a subject without causing any undesirable biological effects. Thus, such a pharmaceutical composition may be used, for example, in transfection of a cell *ex vivo* or in administering a viral particle or cell directly to a subject.

The parvovirus vectors of the invention maybe administered to elicit an immunogenic response (*e.g.*, as a vaccine). Typically, vaccines of the present invention comprise an immunogenic amount of infectious virus particles as disclosed herein in combination with a pharmaceutically-acceptable carrier. An "immunogenic amount" is an amount of the infectious virus particles that is sufficient to evoke an immune response in the subject to which the pharmaceutical formulation is administered. Typically, an amount of about 10^3 to about 10^{15} virus particles, preferably about 10^4 to about 10^{10}, and more preferably about 10^4 to 10^6 virus particles per dose is suitable, depending upon the age and species of the subject being treated, and the immunogen against which the immune response is desired. Subjects and immunogens are as described above.

The present invention further provides a method of delivering a nucleic acid to a cell. For *in vitro* methods, the virus may be administered to the cell by standard viral transduction methods, as are known in the art. Preferably, the virus particles are added to the cells at the appropriate multiplicity of infection according to standard transduction methods appropriate for the particular target cells. Titers of virus to administer can vary, depending upon the target cell type and the particular virus vector, and may be determined by those of skill in the art without undue experimentation. Alternatively, administration of a parvovirus vector of the present invention can be accomplished by any other means known in the art.

Recombinant virus vectors are preferably administered to the cell in a biologically-effective amount. A "biologically-effective" amount of the virus vector is an amount that is sufficient to result in infection (or transduction) and expression of the heterologous nucleic acid sequence in the cell. If the virus is administered to a cell *in vivo* (*e.g.*, the virus is administered to a subject as described below), a "biologically-effective" amount of the virus vector is an amount that is sufficient to result in transduction and expression of the heterologous nucleic acid sequence in a target cell.

The cell to be administered the inventive virus vector may be of any type, including but not limited to neural cells (including cells of the peripheral and central nervous systems, in particular, brain cells), lung cells, retinal cells, epithelial cells (*e.g.*, gut and respiratory epithelial cells), muscle cells, pancreatic cells (including islet cells), hepatic cells, myocardial cells, bone cells (*e.g.*, bone marrow stem cells), hematopoietic stem cells, spleen cells, keratinocytes, fibroblasts, endothelial cells, prostate cells, germ cells, and the like. Alternatively, the cell may be any progenitor cell. As a further alternative, the cell can be a stem cell (*e.g.*, neural stem cell, liver stem cell). Moreover, the cells can be from any species of origin, as indicated above.

In particular embodiments of the invention, cells are removed from a subject, the parvovirus vector is introduced therein, and the cells are then replaced back into the subject. Methods of removing cells from subject for treatment *ex vivo*, followed by introduction back into the subject are known in the art. Alternatively, the rAAV vector is introduced into cells from another subject, into cultured cells, or into cells from any other suitable source, and the cells are administered to a subject in need thereof.

Suitable cells for *ex vivo* gene therapy include, but are not limited to, liver cells, neural cells (including cells of the central and peripheral nervous systems, in particular, brain cells), pancreas cells, spleen cells, fibroblasts (*e.g.*, skin fibroblasts), keratinocytes, endothelial cells, epithelial cells, myoblasts, hematopoietic cells, bone marrow stromal cells, progenitor cells, and stem cells.

Dosages of the cells to administer to a subject will vary upon the age, condition and species of the subject, the type of cell, the nucleic acid being expressed by the cell, the mode of administration, and the like. Typically, at least about 10^2 to about 10^8, preferably about 10^3 to about 10^6 cells, will be administered per dose. Preferably, the cells will be administered in a "therapeutically-effective amount".

A "therapeutically-effective" amount as used herein is an amount of that is sufficient to alleviate (*e.g.*, mitigate, decrease, reduce) at least one of the symptoms associated with a disease state. Alternatively stated, a "therapeutically-effective" amount is an amount that is sufficient to provide some improvement in the condition of the subject.

A further aspect of the invention is a method of treating subjects *in vivo* with the inventive virus particles. Administration of the parvovirus particles of the present invention to a human subject or an animal in need thereof can be by any means known in the art for administering virus vectors.

Exemplary modes of administration include oral, rectal, transmucosal, topical, transdermal, inhalation, parenteral (*e.g.*, intravenous, subcutaneous, intradermal, intramuscular, and intraarticular) administration, and the like, as well as direct tissue or organ injection, alternatively, intrathecal, direct intramuscular, intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Alternatively, one may administer the virus in a local rather than systemic manner, for example, in a depot or sustained-release formulation.

In particularly preferred embodiments of the invention, the nucleotide sequence of interest is delivered to the liver of the subject. Administration to the liver may be achieved by any method known in the art, including, but not limited to intravenous administration, intraportal administration, intrabiliary administration, intra-arterial administration, and direct injection into the liver parenchyma.

Preferably, the cells (*e.g.*, liver cells) are infected by a recombinant parvovirus vector encoding a peptide or protein, the cells express the encoded peptide or protein and secrete it into the circulatory system in a therapeutically-effective amount (as defined above). Alternatively, the vector is delivered to and expressed by another cell or tissue, including but not limited to, brain, pancreas, spleen or muscle.

In other preferred embodiments, the inventive parvovirus particles are administered intramuscularly, more preferably by intramuscular injection or by local administration (as defined above). In other preferred embodiments, the parvovirus particles of the present invention are administered to the lungs.

The parvovirus vectors disclosed herein may be administered to the lungs of a subject by any suitable means, but are preferably administered by administering an aerosol suspension of respirable particles comprised of the inventive parvovirus vectors, which the subject inhales. The respirable particles may be liquid or solid. Aerosols of liquid particles comprising the inventive parvovirus vectors may be produced by any suitable means, such as with a pressure-driven aerosol nebulizer or an ultrasonic nebulizer, as is known to those of skill in the art. *See, e.g.*, U.S. Patent No. 4,501,729. Aerosols of solid particles comprising the inventive virus vectors may likewise be produced with any solid particulate medicament aerosol generator, by techniques known in the pharmaceutical art.

Dosages of the inventive parvovirus particles will depend upon the mode of administration, the disease or condition to be treated, the individual subject's condition, the particular virus vector, and the gene to be delivered, and can be determined in a routine manner. Exemplary doses for achieving therapeutic effects are virus titers of at least about 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^3, 10^{14}, 10^{15} transducing units or more, preferably about $10^8 - 10^{13}$ transducing units, yet more preferably 10^{12} transducing units.

In particular embodiments of the invention, more than one administration (*e.g.*, two, three, four, or more administrations) may be employed to achieve therapeutic levels of gene expression. According to this embodiment, and as described above, it is preferred to use parvovirus vectors

having different antigenic properties for each administration to obviate the effects of neutralizing antibodies. As described above, in particular embodiments of the invention, the hybrid and chimeric parvoviruses of the present invention are administered to circumvent neutralizing antibodies in the subject to be treated or to prevent the development of an immune response in the subject. The subject may be presented with seemingly new virus vectors by packaging the rAAV genome within an array of hybrid or chimeric parvovirus capsids.

The foregoing discussion also pertains to pharmaceutical formulations containing parvovirus capsids and other reagents of the invention as well as methods of administering the same.

In summary, the parvovirus vectors, reagents, and methods of the present invention can be used to direct a nucleic acid to either dividing or non-dividing cells, and to stably express the heterologous nucleic acid therein. Using this vector system, it is now possible to introduce into cells, *in vitro* or *in vivo*, genes that encode proteins that affect cell physiology. The vectors of the present invention can thus be useful in gene therapy for disease states or for experimental modification of cell physiology.

Having now described the invention, the same will be illustrated with reference to certain examples, which are included herein for illustration purposes only, and which are not intended to be limiting of the invention.

Example 1

AAV Vectors

All production of AAV vectors used in these investigations utilized the vector production scheme as described in Ferrari *et al.*, (1997) *Nature Med.* **3**:1295 and Xiao *et al.*, (1998) *J. Virology* **72**:2224. Utilizing a transient transfection procedure, rAAV devoid of adenovirus has been generated. *Id.* This protocol utilizes an adenovirus DNA genome that has been incapacitated for viral replication and late gene expression. The mini Ad plasmid while unable to replicate and produce progeny, is still viable for adenovirus gene expression in 293 cells. Using this construct, the AAV packaging strategy

involving new AAV helper plasmid (pAAV/Ad ACG) and AAV vector DNA (sub 201) has been successfully complemented (Samulski *et al.*, (1989) *J of Virology* **63**:3822). This new construct typically generates rAAV of 10^7 – 10^9/10 cm dish of 293 cells (Xiao *et al.*, (1998) *J. Virology* **72**:2224). Efficient gene delivery is observed in muscle, brain and liver with these vectors in the complete absence of Ad.

Example 2

Cells and Viruses

Human 293 and HeLa cells were maintained at 37°C with 5% CO_2 saturation in 10% fetal bovine serum (Hyclone) in Dulbecco's modified Eagles medium (Gibco BRL), with streptomycin and penicillin (Lineberger Comprehensive Cancer Center, Chapel Hill, NC). Four x10^6 293 cells were plated the day before transfection onto a 10cm plate. Cells were transfected by both calcium phosphate (Gibco BRL) or Superfection (Qiagene) according to manufacturers specifications. The insertional mutant packaging plasmids, described below, were transfected along with pAB11 containing the CMV driven Lac Z gene with a nuclear localization signal. For each transfection the same amount of packaging plasmid (12μg) and pAB11 (8μg) were used for each 10cm plate. For each transfection an additional plate was used containing the transgene plasmid only to assess transformation efficiencies. After transfection the cells were infected with helper virus Ad5 *dl309* at an MOI of 5, and 48 hours later the cells were lysed and the virus purified.

Recombinant virus was purified using cesium chloride isopycnic or iodixanol gradients. In both cases cells were centrifuged at 1500rpms (Sorvall RT 6000B) for ten minutes at 4°C. Proteins were precipitated from the supernatant using ammonium sulfate (30% w/v) and resuspended in 1 x Phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 $7H_2O$, 1.4 mM KH_2PO_4). The cell pellet was resuspended in 1 x PBS containing 0.1 mg/ml DNase I (Boehringer Mannheim) lysed by three freeze-thaw cycles, combined with the protein portion of the supernatant, and incubated at 37°C for 30 minutes. This material was subjected to sonication

(Branson Sonifier 250, VWR Scientific), 25 bursts at 50% duty, output control 2. Cell debris was removed by centrifugation (Sorvall RT 6000B). To each milliliter of supernatant 0.6g of cesium chloride (CsCl) was added and the solution was centrifuged for 12-18 hours (Beckman Optima TLX ultracentrifuge) in a TLS 55 rotor at 55,000 rpms. Alternatively, the supernatant was layered on top of an Iodixanol (OptiPrep -Nycomed Pharma As, Oslo, Norway) gradient of 60%, 45%, 30% and 15%. This gradient was centrifuged in a Beckman Optima TLX ultracentrifuge using a TLN 100 rotor at 100,000 rpm for one hour. Fractions were recovered from these gradients and 10µl from each fraction were utilized for dot blot hybridization to determine which fraction contained the peak protected virion (see **Example 5**).

Example 3

Construction of AAV Packaging Plasmids

The capsid domain of pAAV/Ad was cloned into pBS+ (Stratagene) using *Hind III*, resulting in pAV2Cap. Partial digestion of pAV2Cap using the restriction enzymes *Hae III*, *Nla IV*, and *Rsa I* and gel purification of the unit length DNA fragment resulted in the isolation of the starting material for cloning. The aminoglycoside 3'-phosphotransferase gene, conferring kanamycin resistance (kan^r), from pUC4K (Pharmacia) digested with *Sal I* was flanked by linkers containing *Nae I* and *Eco RV* sites, a *Sal I* overhang at one end and an *Eco RI* overhang at the other end (top 5'-AATTCGCCGGCGATATC-3', **SEQ ID NO:6**, bottom 5'-TCGAGATATCGCCGGC-3', **SEQ ID NO:7**). This fragment was cloned into the *Eco RI* site of pBluescript SK+ (Stratagene). Digestion with *Nae I* released the kan^r gene, and this fragment was ligated into the pAV2Cap partials. The resulting plasmids were screened for insertion into the capsid domain and, then digested with *Eco RV* to remove the kan^r gene leaving the twelve base pair insertion 5'-GGCGATATCGCC-3' (**SEQ ID NO:8**) within the capsid domain. Multiple enzyme digests and DNA sequencing were used to determine the position of the 12bp insertion within the capsid coding domain.

The enzyme digests include *Eco RV/Ban II*, *Eco RV/Bst NI*, *Eco RV/Pst I* and *Eco RV/Hind III*. The capsid domain of the resulting plasmids were digested with *Asp718* and subcloned into the pACG2 packaging plasmid (Li et al., 1997 *J. Virology* **71**:5236), with the exception of one *NlaIV* clone that overlapped the 3'-*Asp718* site. This insertion mutant was cloned into pAAV/Ad using a *Hind III / Nsi I* digestion.

Example 4

Western Blotting

Cell lysates after freeze thaw lysis and sonication was centrifuged to remove large cell debris. Twenty microliters of supernatant was immediately added to 20μl of 2xSDS gel-loading buffer containing dithiothreitol and boiled for five minutes. Proteins were analyzed by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose electrophoretically. The nitrocellulose membranes were immunoblotted using the anti-Vp3 monoclonal antibody B1 (a generous gift from Jurgen A. Kleinschmidt). Each of the insertion mutants was tested at least twice by Western blot analysis. The secondary anti-mouse Horseradish Peroxidase IgG was used to indirectly visualize the protein by enhanced chemiluminescence (ECL-Amersham). The Western blots were scanning from enhanced chemiluminescence exposed BioMax film (Kodak) into Adobe PhotoShop and analyzed by ImageQuaNT software (Molecular Dynamics Inc.).

Viral proteins were visualized by Western blotting followed by immunoblotting as described above. Between 1.0×10^9 and 2.5×10^9 viral particles were used for each sample. The virus was isolated from the peak cesium gradient fraction as determined by dot blot, and dialysed against 0.5 x PBS containing 0.5 mM $MgCl_2$ prior to polyacrylamide gel electrophoresis.

Example 5

Titration of Recombinant Virus

Fractions from CsCl gradients were obtained by needle aspiration. The refractive index was obtained using a refractometer (Leica Mark II), and

the index was used to determine the density of fractions. Aliquots of 10μl from fractions between 1.36g/ml and 1.45g/ml were tested for the presence of protected particles by dot blot hybridization. The aliquots were diluted 1:40 in viral dilution buffer (50mM Tris HCl, 1mM $MgCl_2$, 1mM $CaCl_2$ 10μg/ml RNase, 10μg/ml DNase) and incubated at 37°C for 30 minutes. To the samples Sarcosine (final concentration 0.5%) and EDTA (final 10mM) were added and incubated at 70°C for 10 minutes. Proteinase K (Boehringer Mannheim) was added to a final concentration of 1mg/ml and the samples were incubated at 37°C for two hours. Following this incubation the samples were denatured in NaOH (350mM final) and EDTA (25mM final). The samples were applied to equilibrated nytran (Gene Screen Plus, NEN Life Science Products) using a dot blot manifold (Minifold I, Schleicher and Schuell). The membrane was probed with a random primed (Boehringer Mannheim) ^{32}P-dCTP labeled Lac Z DNA fragment. The membranes were exposed to film (BioMax MR, Kodak) or to phosphor imagining screens (Molecular Dynamics) and intensity estimates were done using ImageQuant software (Molecular Dynamics). Peak fraction of virus were then dialysed in 1 x PBS for transducing titer.

Transductions titers were determined by histochemical staining for Lac Z activity. HeLa cells had been infected with Ad *dl309* at a multiplicity of infection of five for one hour. The cells were then washed with 1 x PBS and fresh medium was added. Aliquots of virus from peak fractions, equivalent to 1.75 x 10^8 particles were used to infect Hela cells. Twenty to twenty-four hours later cells were washed with 1 x PBS, fixed (2% formaldehyde 0.2% gluteraldehyde in 1 x PBS), washed, and stained with 5'-Bromo-4-chloro-3-indoly-β-D-galactopyranoside (Gold Bio Technology) dissolved in N,N-dimethylformamide (Sigma) diluted to 1mg/ml in 1 x PBS pH7.8, 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM MgCl2 at 37°C for 12-24 hours. Stained HeLa cells were counted in ten 400X microscope fields. The transducing number was determined by averaging the number of stained cells in ten fields and multiplying by the number of fields on the plate and dividing that number by the number of nanograms of protected template.

Example 6

Electron Microscopy

Peak fractions of rAAV with wildtype virion or mutagenized virions were dialysed in 0.5 x PBS containing 0.5mM $MgCl_2$. The virus was placed on a 400 mesh glow discharged carbon grid by inverting on a 10µl drop of virus for ten minutes at room temperature. Followed by three 1 x PBS washes for one minute each. The virus was stained in 1% Phosphotungstic acid for one minute. Specimens were visualized using a Zeiss EM 910 electron microscope.

Example 7

Heparin Agarose Binding Assay

Recombinant virus containing wild-type capsids or insertion in the capsids were dialysed against 0.5 x PBS containing 0.5mM $MgCl_2$. One hundred microliters of each virus was bound to 100µl of heparin agarose type 1 (H-6508 Sigma, preequilibrated in twenty volumes of 0.5 x PBS containing 0.5mM $MgCl_2$) at room temperature for one hour in a 1.5ml microfuge tube. After each step, binding washes and elutions samples were centrifuged at 2000rpm (Sorvall MC 12V) for two minutes to collect supernatant. Samples were washed six times with 0.5ml of 0.5 x PBS containing 0.5mM $MgCl_2$, and the supernatant collected. Samples were eluted in three steps of 100µl volumes containing 0.5, 1.0 and 1.5M NaCl in 0.5 x PBS containing 0.5mM $MgCl_2$ and the supernatant collected. For each sample 20µl of supernatant from each step was used for dot blot hybridization. The 100% bound control was an internal standard equivalent to one fifth of each input virus used in the dot blot. The heparin agarose viral mixtures were washed six times with 0.5 X PBS 0.5mM $MgCl_2$ in volumes that resulted in a 1:15625 dilution.

Example 8

Construction of Insertional Mutations in rAAV2

In order to evaluate the role of AAV structural proteins in assembly and infectivity, we generated a collection of capsid linker insertion mutants. A

2.8kb *Hind III* fragment of pAAV/Ad (Samulski *et al.*, (1989) *J. Virology* **63**:3822) containing the sequences coding for the capsid domain of AAV2 was subcloned into pBS+. This plasmid, pAV2Cap, was used for partial digestion with *Hae III*, *Nla IV*, and *Rsa I* to generate a substrate for capsid specific insertions (**Fig. 1**). These three DNA restriction enzymes constitute 43 sites that span across the AAV-2 capsid coding sequence of which only 4 overlap. To efficiently identify clones that contain insertions, a kanamycin resistance gene (Kan^r) flanked by a novel oligo (*Nae I/EcoR V*) was ligated with partially digested, full-length, linearized pAV2Cap (*see* **Example 3** and **Fig. 1**). Using ampicillin and kanamycin selection in *E. coli*, insertion mutants were identified and the Kan^r gene was shuttled out of the capsid coding region by digesting and religation with the nested pair of *Eco RV* sites (*see* **Example 3**). This resulted in a specific linker insertion of 12 base pair (bp) carrying a single copy of the unique *Eco RV* site in the capsid coding sequences. The exact positions of the linker insertion were further refined by restriction enzyme digestions, and in six cases sequencing (data not shown). The position of insertion mutants are identified by the first letter of the enzyme used in the partial digestion followed by the nucleotide position of the restriction site in the AAV2 genome, for example *Nla IV* 4160 would be N4160.

The capsid coding sequence from these mapped insertion mutants were subcloned into the helper vectors pACG2 or pAAV/Ad for biological characterization *in vivo* (**Fig. 1**) (Li *et al.*, (1997) *J. Virology* **71**:5236; Samulski *et al.*, (1989) *J. Virology* **63**:3822). Sequence analysis predicts that this 12 base pair insertion cannot result in a termination codon for any of the 43 insertion sites (**Table 1**). Owing to the random nature of the cut site for the enzymes (*Hae III*, *Nla IV*, and *Rsa I*) with respect to codon frame usage and the degeneracy of the *Nla IV* recognition sequence, the 12 bp linker resulted in the insertion of the amino acids GDIA in frame 1 and AISP in frame 3 for all three enzymes, while insertions in frame 2 resulted in WRYR**H** for *Rsa I*, **G**RYRP for *Hae III*, and both **G**RYRP and GRYR**H** for *Nla IV*. The bolded amino acid in these examples represents missense mutation (**Table 1**). The

mutant helper constructs, pACG2^{IN}, were individually transfected into 293 cells along with an AAV reporter vector, containing the β-galactosidase gene in Adenovirus *dl*309 (MOI=5) infected cells (Li *et al.*, (1997) *J. Virology* **71**:5236). The transfected cells were then assayed for capsid expression and recombinant virus production (*see* **Example 5**; Li *et al.*, (1997) *J. Virology* **71**:5236).

Table 1: Physical Structure and Phenotype of AAV2 Capsid Insertion Mutants

Position[1] inserted	Capsid subunit	Frame[2]	Dot blot[3]	Infectious[4]	Heparin Agarose[5]	Electron Microscope	Phenotype	Amino Acid[6]
H2285	VP1	3	2.8×10^7	-	+	normal	Class II	AISP
R2356	VP1	2	1.4×10^8	+	+	N.D.	Class III	WRYR**H**
N2364	VP1	1	-	-	N.D.	N.D.	Class I	GDIA
H2416	VP1	2	1.4×10^7	-	+	N.D.	Class II	**G**RYRP
H2591	VP1	3	1.4×10^7	+	+	normal	Class III	AISP
H2634	VP2	1	2.8×10^7	-	+	normal	Class II	GDIA
H2690	VP2	3	7.0×10^6	+	+	normal	Class III	AISP
R2747	VP2	3	-	-	N.D.	N.D.	Class I	AISP
H/N2944	VP3	2	1.4×10^6	+*	N.D.	N.D.	ClassII/III	**G**RYRP
N3317	VP3	3	1.4×10^5	-	N.D.	N.D.	Class II	AISP
R3391	VP3	2	-	-	N.D.	N.D.	Class I	WRYR**H**
N3561	VP3	1	-	-	N.D.	N.D.	Class I	GDIA
H3595	VP3	2	1.4×10^6	+*	N.D.	abnormal	Class II/III	**G**RYRP
H/N3761	VP3	3	1.4×10^7	-	-	normal	Class II	AISP
H3766	VP3	2	2.8×10^7	-	N.D.	N.D.	Class II	**G**RYRP
N4046	VP3	3	-	-	N.D.	N.D.	Class I	AISP
H/N4047	VP3	1	-	-	N.D.	N.D.	Class I	GDIA
N/R4160	VP3	3	1.4×10^7	+	+	normal	Class III	AISP

1. The letter refers to the restriction enzyme used in the partial digestion and the number refers to nucleotide of the restriction site in the AAV2 sequence.
2. Reading frame of the restriction site.
3. The particle number per microliter of sample. (-) = $<10^5$ genomes.
4. Infections were done using 1.75×10^8 particles of rAAV insertion mutants in adenovirus infected HeLa cells.
5. By batch binding and assayed by infection of HeLa cells (Class III) or by dot blot (Class II).
6. Amino acids differ depending on the frame of the insertion. The bolded amino acid is a missense mutation.

Example 9

Analysis of Capsid Proteins

Before assaying for vector production using mutant capsid constructs in complementation assays, each insertion mutant was tested for expression of capsid subunits in 293 cells after transfection. The ability to produce Vp1, Vp2, and Vp3 at normal stoichiometry would suggest that linker insertions did not alter capsid protein expression, or stability. Since the linker did not introduce stop codons, it was expected that each insert would produce all three capsids. Forty-eight hours after transfection, cell lysates were analyzed by Western blot for AAV capsids. The Western blot analysis in **Figure 2** is a representation of insertion mutant capsid expression in cell lysates. With the exception of H2634 (**Fig. 2** lane 2), the stoichiometry of the three capsid subunits does not appear significantly different than that of wild-type controls (**Fig. 2** compare lanes 1,3-7 to lane 8). By this assay, insertion mutant H2634 appears to only produce Vp3 subunits (**Fig. 2**; lane 2). In longer exposures, the minor capsid subunits in **Figure 4** lanes 4 and 5 were apparent (data not shown).

Example 10

Mutant Capsid Ability to Produce Stable Virions

To test for the production of stable virions that protect a vector genome from DNase digestion, we subjected the cell lysates to cesium chloride (CsCl) gradient centrifugation. Virus densities were measured by refractometry, and aliquots from appropriate fractions were subjected to dot blot hybridization (**Fig. 3a**). Based on this analysis, particles that package intact recombinant genomes should display a buoyant density similar to wild-type and be resistant to DNase treatment, with the exception of H2944 which has a buoyant density slightly higher than wild type. Results for this assay separated insertion mutants into two classes. Class I mutants were negative

for protecting the viral genome, while class II mutants appeared normal for packaging and protecting the vector substrate (**Table I**).

All class II mutants had a buoyant density within the range of wild-type AAV2 capsids (**Fig. 3a**). By dot blot analysis, N2944 packaged the recombinant genome but migrated to a position of slightly greater density than wild type in isopycnic gradients (**Fig. 3a**, N2944 lane 3). A number of insertion mutants (7) did not package DNA by this assay which had a sensitivity of $<1x10^5$ particles/μl (see methods for quantitation) (**Table 1**). Whether these mutants were defective in packaging or unstable during purification remains to be determined.

Example 11

Infectivity of Class II Insertion Mutants

Virions generated by insertion mutants in the complementation assay were tested for infectivity by monitoring transduction of LacZ reporter gene in human cells. Using viral titers derived from dot blot hybridization, HeLa cells were infected with mutant virus stocks at equivalent particle numbers.

Twenty-four hour post infection, expression of the transgene was detected by X-gal staining. A representative figure of this analysis is shown (**Fig. 3b**) and all mutants assayed are presented in **Table 1**. In this assay, wild-type virions transduced $5.6x\ 10^5$ HeLa cells/$1.75\ x10^8$ protected particles (**Fig. 3b**). Based on the sensitivity of this assay, the range of infection efficiency for class II insertion mutant viruses was from 0 to 1.6×10^6 transducing units/1.75×10^8 protected particles. Results from this analysis further subdivided the capsid insertion mutants from class II (normal for packaging and protecting the vector substrate) into a class III phenotype (normal for packaging and protecting the vector substrate and infectious virions). Two insertion mutants negative for infectivity and initially identified as class II mutants (N2944, H3595) based on CsCl purification and DNase protection, tested positive for viral transduction after purification using an iodixanol step gradient (**Table 1**). This virus purification technique is not as harsh as CsCl and has been shown to increase virus recovery by ten-fold

(Zolotukhin *et al.*, (1999) *Gene Therapy* **6**:973). However, other class II mutants remained non-infectious after purification using an iodixanol step gradient (data not shown). Although we determined that insertion mutant viruses N2944 and H3595 were infectious using the Lac Z transduction assay, it should be noted that these mutants resulted in low infectious titers (1 x 10^2 transducing units/ng) similar to previously published *lip* mutants (Hermonat *et al.*, (1984) *J. Virology* **51**:329).

Example 12

Electron Microscopy of Class II and Class III Mutants

To further characterize class II and III rAAV2 insertion mutants for biological differences, we visualized mutant particles by electron microscopy (EM). The EM analysis revealed only gross morphology of the infectious class III viruses, which were indistinguishable from wild-type virions (Compare **Fig. 4a**, and **4b,c**). Whereas distinct differences were observed between class II/III mutant virus H3595 when compared to wild-type virions (**Fig. 4a**, and **4f**-bottom four panels). EM images of H3595 revealed a slightly larger roughly pentagonal outline, while wild-type virus appeared uniformed in size and was hexagonal. Interestingly, class II mutant H2634, which was negative for Vp1 or Vp2 by Western blot (**Fig. 2** lane 2), appeared normal in morphology by EM analysis (**Fig. 4d**). Based on this analysis, virion morphology alone is not sufficient to distinguish class II mutants from class III since small insertions within the capsids can result in either non-detectable (**Fig. 4 b,c,d,e**) or noticeable alterations in virion structure (**Fig. 4f**-bottom four panels). However, this approach was able to provide additional data to our characterization of these linker insertion mutants (**Fig. 4**, compare a to f).

Example 13

Capsid Ratio of Class II and Class III Virions

Rose et. al.(1971) established that AAV2 particles are composed of Vp1, Vp2, and Vp3 at a 1:1:20 ratio (Rose *et al.*, (1971) *J. Virology* **8**:766). In an effort to determine if class II and class III mutant virions maintained this

ratio, Western blots were performed on the cesium chloride purified virus. Purified viruses analyzed by Western blot showed similar amounts of Vp3 in all mutants sampled (**Fig. 5**, Vp3 arrow), between 1 $x10^9$ and 2.5 $x10^9$ viral particles were used for each sample. The amounts of Vp2 and Vp1 are also nearly equivalent in all test samples except H2634 where no minor capsid components were observed (**Fig. 5**, lane 5). The lack of minor capsid components for H2634 is consistent with the Western results from cell lysate (**Fig. 2**). At the limit of detection in this assay, the class II insertion mutant H2634 appears to assemble AAV virions without Vp1 and Vp2, even though EM analysis suggest this mutant has normal morphology (**Fig. 4d**).

Example 14

Heparin Binding of Class II and Class III Mutants

Recently our lab established that AAV-2 uses a heparan sulfate proteoglycan as a primary receptor for infectivity (Summerford and Samulski, (1998) *J. Virology* **72**:1438). To determine what role heparin binding may have in class II particles inability to infect cells as well as the ability of class III virus to bind heparin agarose, heparin batch binding experiments were performed. Not surprisingly, all class III mutants were positive for heparin binding, with the majority of virus eluting in the 1M $NaCl_2$ step (data not shown). To determine if loss of infectivity of class II mutant viruses was related to a lack of heparin binding, batch binding experiments were analyzed by dot blot hybridization (**Fig. 6**). For each of the viral samples tested, an internal control to determine 100% bound was spotted on the filter independent of heparin binding (**Fig. 6**; 100% bound). This allowed us to determine percent virus retained, at each step of heparin purification. After binding to heparin agarose, samples were washed then eluted using increasing salt concentrations (*see* **Example 7**). Recombinant AAV2 with wild-type virion shells demonstrated 90% binding with 10% released in the wash followed by 60% recovered in the elution buffer, and 20% remaining bound to heparin agarose (**Fig. 6**, lane 1). Class II mutants H2285, H2416, and H2634 demonstrated similar binding and elution profiles (**Fig. 6**, lanes 2-

4). However, class II mutant H3761 was distinct in its heparin agarose binding profile with the majority of the virion in the binding buffer and the washes (**Fig. 6**, lane 5). Further analysis is required to determine the reason for lack of Heparin binding in this batch assay.

Interestingly, H2634 binds heparin agarose under these conditions, which by Western blot does not carry detectable Vp1 or Vp2 subunits (**Fig. 5**, lane 4). The lack of Vp1 and Vp2 in H2634 along with its ability to bind heparin agarose suggest that the heparin binding domain may be located in Vp3 capsid proteins.

Example 15

Linker Insertion Mutants

Insertion sequences encoding poly-lysine, poly-histidine, an RGD motif, or bradykinin were inserted into the linker mutants described in **Table 1**. We developed a PCR-based method of identifying insertions of different linkers into the coding domain of AAV2 capsid gene. Briefly, one primer was used outside of the capsid coding region and one that corresponds exactly to the linker. If the linker is in the correct orientation, then the PCR product is of a size that is dependent on the insertion mutant's position.

After transformation of the ligation reactions, bacterial colonies were picked with a pipet tip and dipped 4-5 times into a well of a 96-well plate containing LB-medium with antibiotic. The pipet tip was then placed in a well of a 96-well plate containing PCR reaction buffer. The PCR products were run out on an agarose gel, and positive clones were identified. This information indicated the orientation and the position of the insertion mutant with respect to the outside primer.

The LB-medium that is in the corresponding well was used as the PCR positives, and this material was grown in a larger (5mL) volume. After an overnight growth phase, the plasmid DNA was isolated and digested with an enzyme that restricts the DNA 15 times (Bst NI). These digestion products were separated on a 5-6% acrylamide gel. Depending upon the size of the linker insertion and the size of the corresponding uninserted fragment, the

number of inserts is determined. Thus, within two days of ligating the linker into the insertion site, we know the orientation and number of linker insertion, and we have sufficient DNA to transfect a 10cm plate for virus production.

pACG2 (Li et al., 1997 *J. Virology* **71**:5236) without any insertion when digested with Bst NI yields fragments of:

3900 bp
1121 bp
1112 bp
445 bp -H2944 shifts
347 bp -H2634, H2690 shifts
253 bp -H3595 shifts
215 bp -R2356, H2416 shifts
121 bp
111 bp
64 bp
63 bp -H2285 shifts
33 bp -H2591 shifts
13 bp
9 bp

The band shifts with the different insertion mutants are also indicated.

pACG2 without any insertion when digested with Ban I yields fragments of:

2009 bp
1421 bp
168 bp
843 bp - H4047 shifts
835bp
734bp - H2634 shifts
464bp
223bp
218bp

211bp

50bp

Each of the inserts contains the original 12 base pairs of the Eco RV site. In addition, each of the linkers adds additional base pairs:

- RGD= 36bp + 12 = 48bp for a single insertion.
- Bradykinin (BRDY) = 69bp +12 = 81bp for a single insertion. Note: The BRDY insert contains a BstNI site.
- Histidine (8HIS) = 51bp +12 = 63bp for a single insertion.
- Poly Lysine (PLY) = 63bp +12 =75bp for a single insertion.

The outside primer is near the Hind III site and is called AAV2/4 5'. This primer can be used to amplify AAV serotypes 2 and 4.

Primer sequences used to produce epitope linkers into the original insertion mutants are given below. Note: Because there are three frames for the insertion mutants there are three primer pairs for each primer set.

Histidine primer pairs:

Frame 1:

Top primer a 48mer (**SEQ ID NO:9**):

5' -GCT AGC GGC GGA CAC CAT CAC CAC CAC CAT CAC CAC GGC GGA AGC GCT- 3'

Bottom primer a 48mer (**SEQ ID NO:10**):

5' -AGC GCT TCC GCC GTG GTG ATG GTG GTG GTG ATG GTG TCC GCC GCT AGC- 3'

Frame 2:

Top primer a 51mer (**SEQ ID NO:11**):

5' -AC GCT AGC GGC GGA CAC CAT CAC CAC CAC CAT CAC CAC GGC GGA AGC GCT T- 3'

Bottom primer a 51 mer (**SEQ ID NO:12**):

5' -A AGC GCT TCC GCC GTG GTG ATG GTG GTG GTG ATG GTG TCC GCC GCT AGC GT- 3'

Frame 3:

Top primer a 51mer(**SEQ ID NO:13**):

5' -G GGT TCC GGA GGG CAC CAC CAT CAC CAC CAC CAT CAC GGA GGC GCC AGC GA- 3'

Bottom primer a 51mer (**SEQ ID NO:14**):

5' -TC GCT GGC GCC TCC GTG ATG GTG GTG GTG ATG GTG GTG CCC TCC GGA ACC C- 3'

Bradykinin primer pairs:

Frame 1:

Top primer a 60mer (**SEQ ID NO:15**):

5' -GCC GGA TCC GGC GGC GGC TCC AGA CCC CCC GGC TTC AGC CCC TTC AGA TCC GGC GGC GCC- 3'

Bottom primer a 60mer (**SEQ ID NO:16**):

5' -GGC GCC GCC GGA TCT GAA GGG GCT GAA GCC GGG GGG TCT GGA GCC GCC GCC GGA TCC GGC- 3'

Frame 2:

Top primer a 69mer (**SEQ ID NO:17**):

5' -GA GGT TCA TGT GAC TGC GGG GGA AGA CCC CCT GGC TTC AGC CCA TTC AGA GGT GGC TGC TTC TGT GGC G- 3'

Bottom primer a 69mer (**SEQ ID NO:18**):

5' -C GCC ACA GAA GCA GCC ACC TCT GAA TGG GCT GAA GCC AGG GGG TCT TCC CCC GCA GTC ACA TGA ACC TC- 3'

Frame 3:

Top primer a 60mer (**SEQ ID NO:19**):

5'-A GGT TCA TGT GAC TGC GGG GGA AGA CCC CCT GGC TTC AGC CCA TTC AGA GGT GGC TGC TTC TGT GGC GG- 3'

Bottom primer a 60mer (**SEQ ID NO:20**):

5' -CC GCC ACA GAA GCA GCC ACC TCT GAA TGG GCT GAA GCC AGG GGG TCT TCC CCC GCA GTC ACA TGA ACC T- 3'

RGD primer pairs:

Frame 1:

Top primer a 36mer (**SEQ ID NO:21**):

5' -GGA TCC TGC GAC TGC AGG GGC GAT TGT TTC TGC GGC- 3'

Bottom primer a 36mer (**SEQ ID NO:22**):

5' -GCC GCA GAA ACA ATC GCC CCT GCA GTC GCA GGA TCC- 3'

Frame 2:

Top primer a 36mer (**SEQ ID NO:23**):

5' -GA TCC TCG GAC TGC AGG GGC GAT TGT TTC TGC GGC G- 3'

Bottom primer a 36mer (**SEQ ID NO:24**):

5' -C GCC GCA GAA ACA ATC GCC CCT GCA GTC GCA GGA TC- 3'

Frame 3:

Top primer a 36mer (**SEQ ID NO:25**):

5' -A GGA TCC TGC GAC TGC AGG GGC GAT TGT TTC TGC GG- 3'

Bottom primer a 36mer (**SEQ ID NO:26**):

5' -CC GCA GAA ACA ATC GCC CCT GCA GTC GCA GGA TCC T- 3'

Polylysine primer pair:

Note: only the frame three primer pair was made.

Frame 3:

Top primer a 63mer (**SEQ ID NO:27**):

5' -A GGT TCA TGT GAC TGC GGG GGA AAG AAG AAG AAG AAG AAG AAG GGC GGC TGC TTC TGT GGC GG- 3'

Bottom primer a 63mer (**SEQ ID NO:28**):

5' -CC GCC ACA GAA GCA GCC GCC CTT CTT CTT CTT CTT CTT CTT TCC CCC GCA GTC ACA TGA ACC T- 3'

Outside primer AAV 2/4 5' top primer (**SEQ ID NO:29**):

5' -TGC CGA GCC ATC GAC GTC AGA CGC G- 3'

The RGD linker was inserted into the H2285, R2356, H2591, H2634, H2690, H/N3761, and H/N4047 mutants from **Table 1**.

The bradykinin linker was inserted into the H2285, H2416, H2591, H2634, H2690, H/N2944, and H/N3761 mutants from **Table 1**.

The poly-Lys linker was inserted into the H2285, H2591, H2690, and H/N3761 mutants from **Table 1**.

The poly-His linker was inserted into the H2285, H2416, H2591, H2634, H2690, H/N2944, N3561, H3766, and H/N4047 mutants from **Table 1**.

Example 16

Characterization of Insertion Mutants

The insertion mutants at site H2690 all have titers similar to the original 12bp insert. Using the ELISA assay and the anti-histidine antibody

polyHis insertions into this site were shown to be displayed on the surface of the virion.

The polyHis epitope was also shown to be on the surface when inserted into site H2634. Interestingly, the Western blot analysis of the 12bp insertion at H2634 did not show any VP1 or VP2 subunits being formed. It has been determined that this insertion in VP2 is near the nuclear localization signal for the VP1 And VP2 subunits. It is possible that this domain was disrupted by the original insertion, and with the addition of the 8-histidines the domain was repaired. Although the dot blot of this 8His virus showed the presence of viral particles, these particles were not infectious.

The insertion site H2591 is in VP1. Insertion of linker epitopes into this site do not affect the titer any more than did the original 12bp insertion at this site (**Table 1**).

The insertion at site N4160 is in VP3 near the carboxy terminus. This insertion mutant is of interest because the original 12bp insertion infects cell at an equivalent level as wild-type (**Table 1**).

Mutant R3317, which has been previously described in Table1, appeared not to protect virions by dot blot analysis. Repeating this experiment with a LacZ transgene, the same results were observed, *i.e.*, no protected particles. However, when using an independent clone and the GFP transgene (~1000bp smaller than LacZ) protected particles were observed. In addition, the GFP-expression virion transduced HeLa cells at high levels, equivalent to wild-type. It is unclear why disparate results were observed with different transgenes.

In addition, a linker encoding the respiratory syncitial virus heparin binding domain is inserted into the H2690 mutant at a site that tolerates inserts without loss of viability (**Table 1**) to restore heparin binding to this mutant.

Example 17

Unique Restriction Site Mutants

Unique restriction sites within the capsid of AAV type 2 were made to

facilitate the generation of insertional mutants. The sites were chosen so that the mutations introduced into the nucleotide sequence of the capsid were conservative, *i.e.*, were not missense mutations or result in stop codons. Amino acid positions 586, 529, 595, 552, and 517 (VP1 methionine as amino acid #1) were chosen. For all of these positions, except 529, unique Hpa I sites were engineered. For the site at amino acid 529, a unique Eco RV site was engineered. Each of these unique restriction sites results in an in-frame blunt ended digestion product. So frame 1 linkers were used to insert into these sites. Overlapping primers were used to generate the unique sites, and outside primers were used to generate the right and left fragments of the insertion.

The right fragment was then digested with Nsi I and either Eco RV or Hpa I, and the left fragment with Hind III and either Eco RV or Hpa I. We cloned these digestion products into the pACG vector that had already digested with Hind III and Nsi I. The resulting plasmid was then digested with Xcm I and Bsi WI. These enzymes result in an ~750 bp fragment around the engineered unique restriction site. This strategy will result in the accumulation of fewer errors because the PCR generated sequences are smaller.

The primers:

595 top primer (**SEQ ID NO:30**):
5' -GCA GAT GTT AAC ACA CAA GGC GTT CTT CCA- 3'

595 bottom primer (**SEQ ID NO:31**):
5' -TTG TGT GTT AAC ATC TGC GGT AGC TGC TTG- 3'

586 top primer (**SEQ ID NO:32**):
5' -CAG AGA GTT AAC AGA CAA GCA GCT ACC GC- 3'

586 bottom primer (**SEQ ID NO:33**):

5' -GTC TGT TAA CTC TCT GGA GGT TGG TAG ATA- 3'

Note: This construct results in a missense mutation Glycine to Valine

552 top primer (**SEQ ID NO:34**):
5' -ACA AAT GTT AAC ATT GAA AAG GTC ATG ATT- 3'

552 bottom primer (**SEQ ID NO:35**):
5' -TTC AAT GTT AAC ATT TGT TTT CTC TGA GCC- 3'

529 top primer (**SEQ ID NO:36**):
5' -GGA CGA TAT CGA AAA GTT TTT TCC TCA G- 3'

529 bottom primer (**SEQ ID NO:37**):
5' -ACT TTT CGA TAT CGT CCT TGT GGC TTG C- 3'

Note: This construct results in a missense mutation Glutamic acid to Isoleucine

517 top primer (**SEQ ID NO:38**):
5' -TCT CTG GTT AAC CCG GGC CCG GCC ATG GCA- 3'

517 bottom primer (**SEQ ID NO:39**):
5' -GCC CGG GTT AAC CAG AGA GTC TCT GCC ATT- 3'

The outside primers were:
5' primer (**SEQ ID NO:40**):
5' -TGC GCA GCC ATC GAC GTC AGA CGC G- 3'

3' primer (**SEQ ID NO:41**):
5' -CAT GAT GCA TCA AAG TTC AAC TGA AAC GAA T- 3'

Four clones were also generated with the RGD and 8His linkers (**Example 15**) inserted into the 529 Eco RV site. Five 8His linkers and one RGD linker insertion mutants were generated into the 586 Hpa I site.

The unique restriction site missense mutations at 3790-3792 (amino acid 529; EcoRV) did infect HeLa cells, although at relatively low efficiency (~1/100 to ~1/1000 of wild-type). When the 8His epitope insert was inserted at this site, the resulting virus had a *lip* phenotype (*i.e.*, a low infectious particle).

Insertions into the unique missense restriction site at 3960-3961 (amino acid 586; Hpa I) both 8His and RGD were both very infectious, transducing HeLa cells at least as well as wild-type virus.

Example 18

Double Mutants

Double mutants were generated using the single mutant H3761 (**Table 1**) as a template. The H3761 insertion mutant does not bind heparin sulfate as assessed by both batch and column binding experiments. This mutant is interesting because it does not infect any of the cell lines so far tested, although electron microscopy analysis suggests that this virus forms normal parvovirus shells, and by dot blot hybridization this virus packages the viral genome efficiently.

The region of the capsid coding the sequence that contains the H3761 insertion was subcloned into other insertion mutants to create double-mutants. The H2690 (AA# 163) insertion mutant was chosen because it has been shown to display a poly-His insertion epitopes on the viral surface (as assessed by using the conformational specific antibody to bind the virus to an ELISA plate and an anti-histidine antibody preconjugated to horse radish peroxidase to detect the virus containing histidines).

The H2690 insertion mutant helper plasmid (pACG H2690 BRDY) containing the bradykinin insertion (**Example 15**) and the pACG H3761 insertion mutant were both digested with Hind III and Bsi WI. The Hind III site is in the rep gene, while the Bsi WI site is between 2690 and 3761. The small

fragment contains pACG H2690 BRDY while the large fragment contains pACG H3761.

A double mutant H2690 BRDY H3761, with the bradykinin insert inserted at the H2690 site, demonstrated a five-fold increase in infectivity of A9 cells expressing the bradykinin receptor as compared with the parental A9 cells alone. These results indicate (1) the defect in binding of the H3761 is likely at the point of binding to cellular HS receptors, but this virus retains infectivity if directed into cells by another route, and (2) the bradykinin double-mutant targeted entry of the virus into bradykinin-receptor expressing cells.

The H3761 insertion mutant has also been cloned into the unique restriction site missense mutations (**Example 17**), AA# 586 (Hpa I) and AA# 529 (EcoRV). The restriction enzyme NcoI lies between the H3761 and the 529 (Glu →Ile) and 586 (Gly →Val) missense mutations, and this enzyme cuts within the rep gene. By digesting the pACG2 helper plasmid contain the H3761 and the 586 and 529 unique sites with Nco I, the small Nco I fragment (3142bps) containing the H3761 insertion mutation and the large Nco I fragment (5034 bps) containing the 586 and 529 unique sites were isolated. After ligation, the constructs with the correct orientation were established, and these clones were used to make virus.

The unique restriction site missense mutations that containing the RGD motif (**Example 15**) were also used in this cloning strategy. Thus, there are double mutants containing no inserts at the unique sites and double mutants containing RGD epitopes at those sites.

The H3761 mutant does not transduce HeLa or CHO-K1 cells. In contrast, the 586-RGD double mutants exhibited transduction of both of these cell types. These results strongly suggest that the transduction was mediated by the RGD motif introduced into the 586 unique restriction site.

The double mutants with the unique restriction sites, but no inserts, and the 529-RGD double mutant did not exhibit efficient transduction of HeLa or CHO-K1 cells.

Example 19

MSH-Targeted AAV Vector

In one embodiment of the invention, melanocyte stimulating hormone (MSH) is used for targeting of AAV vectors to cells expressing MSH receptors. Studies have shown that this peptide will direct ligand-associated complexes specifically into melanocyte NEL-M1 cells (Murphy *et al.*, (1986) *Proc. Nat. Acad. Sci USA* **83**:8258), providing a convenient test system. For example, diphtheria toxin tethered to a 12-residue peptide encoding the MSH ligand was efficient in killing only MSH receptor expressing cells (Morandini *et al.*, (1994) *Internat. J. Ca.* **56**:129). Cell death was attributed to receptor mediated endocytosis of the specific ligand delivery.

MSH is inserted into loop 3 of the AAV type 2 capsid. In the first step, an AAV type 2 deletion mutant is made with a 12-amino acid deletion when the Bgl II – SpH I fragment is removed from the sequence encoding loop 3. The sequence encoding the MSH peptide is then inserted into the deleted region.

The primer sequences to make the loop3 and loop4 insertion mutations are as follows:

Loop 3 5' top primer (**SEQ ID NO:42**):

5-'GATACTTAAGATCTAGTGGAACCACCACGCACTCAAGGCTT-3'

The cttaag is an Afl II site, the agatct is a Bgl II site. These two sites overlap by two base pairs. The homology with the AAV sequence starts at position 3556 and ends at 3583.

Loop 3 3' bottom primer (**SEQ ID NO:43**):

5'-CTAGCTTAAGCATGCATACAGGTACTGGTCGATGAGAGGATT -3'

The gcatgc is a SphI site, and the cttaag is an Afl II site. These two sites overlap by one base pair. The homology with the AAV sequence starts at position 3505 and ends at 3531 (note that this is the bottom strand).

These primers remove 24 bp (*i.e.*, 8 amino acids) of AAV type 2 sequences from 3532 to 3555. The deleted amino acid sequence is Tyr Leu Ser Arg Thr Asn Thr Pro from at amino acid 444 to 451 (VP1-Met being amino acid #1).

The 5'Sph I Afl II Bgl II 3' sites in the sequence: 5'-GCATGCTTAAGATCT-3' result in the addition of 5 amino acids Ala Cys Leu Arg Ser.

Virus is produced by standard packaging methods. The MSH-tagged AAV type 2 vector is evaluated for transduction in HeLa cells and cells with MSH receptors (*e.g.*, melanocytes).

Example 20

Chimeric AAV2/4 Virus –Capsid Protein Substitutions

The virions of the AAV serotypes are made up of three protein subunits VP1 VP2 and VP3. VP3 is the most abundant subunit, it represents between 80-90% of the 60 subunits that make up the virion, with VP1 and VP2 making up 5-10% each of the virion. The subunits are translated from an overlapping transcript, so that VP3 sequences are within both VP2 and VP1, and VP2 sequences are within VP1.

We have designed primers that enabled us to substitute entire subunits and unique domains of subunits between AAV2 and AAV4. AAV4 has properties that are significantly different from AAV2. Thus, defining the domains that account for these distinct properties would be of value, *e.g.*, for designing gene therapy vectors.

We have chosen a seamless cloning strategy to clone the subunits or unique domains of subunits between these two serotypes.

AAV2 and AAV4 top primer (**SEQ ID NO:44**):
5' -TGC CGA GCC ATC GAC GTC AGA CGC G- 3'

AAV2 and AAV4 bottom primer (**SEQ ID NO:45**):
5' -CAT GAT GCA TCA AAG TTC AAC TGA AAC GAA T- 3'

AAV2 VP3 top primer (**SEQ ID NO:46**):
5' -CGA GCT CTT CGA TGG CTA CAG GCA GTG GCG CAC- 3'

AAV2 VP3 bottom primer (**SEQ ID NO:47**):
5' -AGC GCT CTT CCC ATC GTA TTA GTT CCC AGA CCA GAG- 3'

AAV2 VP2 top primer (**SEQ ID NO:48**):
5' -CGA GCT CTT CGA CGG CTC CGG GAA AAA AGA GGC- 3'

AAV2 VP2 bottom primer (**SEQ ID NO:49**):
5' -AGC GCT CTT CCC GTC TTA ACA GGT TCC TCA ACC AGG- 3'

AAV4 VP3 top primer (**SEQ ID NO:50**):
5' -CGA GCT CTT CGA TGC GTG CAG CAG CTG GAG GAG CTG- 3'

AAV4 VP3 bottom primer (**SEQ ID NO:51**):
5' -AGC GCT CTT CGC ATC TCA CTG TCA TCA GAC GAG TCG-3'

AAV4 VP2 top primer (**SEQ ID NO:52**):
5' -CGA GCT CTT CGA CGG CTC CTG GAA AGA AGA GAC- 3'

AAV4 VP2 bottom primer(**SEQ ID NO:53**):
5' -AGC GCT CTT CCC GTC TCA CCC GCT TGC TCA ACC AGA- 3'

These primers will result in the subunit swaps that are shown in **Figure 7**. A representative sequence of a chimeric AAV2 capsid in which the AAV4 Vp2 was substituted is shown in **Appendix 2** (**SEQ ID NO:2**). This sequence contains the AAV2 rep coding sequences, most of the AAV2 Vp1 and Vp3 coding sequences, and the entire AAV4 Vp2 coding sequences and some of the AAV4 Vp1 and Vp3 coding sequences in a pBluescript backbone.

The Rep68/78 coding sequence begins at nu 251 of **SEQ ID NO:2**, and the Rep52/40 coding sequence begins at nu 923. The Rep78/52 stop signal ends at nu 2114, and the stop for Rep68/40 is at nu 2180. The capsid coding sequence starts at nu 2133 and the end at nu 4315 (Vp1 start at nu 2133, Vp2 start at nu 2544, Vp3 start at 2724).

The AAV2 sequences from the second XhoI site at bp 2420 in Vp1 to the Bsi WI site at bp 3255 in Vp3 in the AAV2 cap genes was replaced with the corresponding region from AAV4 (corresponding to nu 2350-3149 in the plasmid sequence). Briefly, the AAV2 helper plasmid pACG2 was partially digested with XhoI and Bsi WI releasing the 835 bp fragment. The same digest in AAV4 resulted in a 799 bp fragment that was ligated into the deleted AAV2 sequence to produce the helper virus encoding the chimeric AAV2/4 capsid.

Virions are produced carrying a recombinant AAV genome, preferably a recombinant AAV2 genome, typically expressing a reporter gene (*e.g.*, GFP). These mutant viral vectors are characterized for virion formation, morphology, genome protection, heparin binding, and infectivity as described in **Example 15**.

Example 21

Construction of B19/AAV-2 Chimeric Vectors

Studies by Dong *et al.*, (1996) *Human Gene Therapy* **7**:2101, have determined the packaging limitations using rAAV vectors. Using recombinant AAV DNA templates with increasing insertions of stuffer DNA, Dong *et al.* determined that the packaging capacity of rAAV vectors declined dramatically between 104% and 108% of wt (4883 vs. 5083 nucleotides, respectively). This packaging restriction precludes the use of important genes, including mini muscular dystrophy genes as well as promoter regulated cystic fibrosis sequences.

Accordingly, the present investigations set out to develop a B19/AAV-2 derived gene therapy vector that maintains the packaging capacity of B19, the tropism of AAV-2, as well as function as a substrate for targeting vectors.

The human parvovirus B19 (packaging capacity of 5.6 kb) was chosen to utilize the major structural protein Vp2 in the generation of a chimeric AAV vector for packaging larger vector genomes. B19 is composed of only two overlapping structural proteins (Vp1 & 2). B19 infects primary erythroid progenitor cells using globoside as its receptor (Brown *et al.*, (1993) *Science* **262**:114). The structure of B19 has been determined to 8 Å resolution (Agbandje-McKenna *et al.*, (1994) *Virology* **203**:106).

A chimeric AAV particle was constructed by swapping the AAV major structural protein Vp3 for B19's Vp2. Seamless cloning (Stratagene USA) was utilized to generate an AAV helper construct that would express all of the AAV proteins (Rep 78, 68, 52, 40 and Vp 1 and Vp2) with B19 substituted for the Vp3 major Cap protein (**Figure 8**; nucleotide sequence in **Appendix 3** and **SEQ ID NO:3**; amino acid sequence in **Appendix 4** and **SEQ ID NO:4**).

The starting material for the chimeric vector was pAAV-Ad and pYT103c. pYT103c contains the entire B19 coding domain without terminal repeats. HindIII digestion of pAAV-Ad released a 2727bp fragment which contained the entire AAV2 capsid coding region and some flanking regions. This fragment was subcloned into Hind III digested pBS+(Stratagene), resulting in pBS+AAVCap. Polymerase chain reaction was used to amplify the Vp2 coding region from pYT103c. The primers were 5'-AGTTACTCTTCCATGACTTCAGTTAATTCTGCAGAA 3' (**SEQ ID NO:54**) in the 5' direction and 5'- AGTTACTCTTCTTTACAATGGGTGCACACGGCTTTT 3' (**SEQ ID NO:55**) in the 3' direction. Primers to pBS+AAVCap were used to amplify around Vp3 of AAV2. The primers were 5'-AGTTACTCTTCTTAATCGTGGACTTACCGTGGATAC 3' (**SEQ ID NO:56**) in the 5' direction and 5'-AGTTACTCTTCCCATCGTATTAGTTCCCAGACCAGA 3 (**SEQ ID NO:57**) , in the 3' direction. Six nucleotides from the 5' end of each primer is an Eam 1104 I site, this site digests downstream from its recognition site in this case the overlap is an ATG and its compliment and a TAA and its compliment. This site is utilized during the seamless cloning strategy (Stratagene). Digestion of B19-Vp2 and AAV2 PCR products with Eam 1104-I and cloning resulted in a subclone of pBS+AAVCap with Vp2 of

B19 substituted for AAV2 Vp3. This vector was digested with Hind III and cloned back into pAAV-Ad and orientation determined resulting in pAAV/B19-Ad (**Appendix 3; SEQ ID NO:3**). This sequence encodes the AAV2 Vp1 region (start at nt 1), followed by the AAV2 Vp2 region (start at nt 412), and then the B19 Vp2 region (start at nt 607).

Example 22

Production of Chimeric Virus

The pAAV/B19 helper construct was used in a transient packaging system as described in **Example 1**. Briefly, the helper plasmids pAAV/B19-Ad and pAB11 (which contains AAV2 terminal repeats and the β-galactosidase gene under the control of the CMV early promoter) were co-transfected into 293 cells by calcium phosphate. Twelve hours after transfection the medium was changed and adenovirus dl309 (MOI-5) was added. Forty-eight hours later the cells were centrifuged and the supernatant was discarded. A fraction of the cell pellet was used in a HIRT assay. The cell pellet was lysed in cesium chloride (1.39 g/ml), sonicated and centrifuged at 41,000 rpm for 72 hours. Fractions from the cesium gradient were recovered and samples from each were used in dot blot hybridization to test particle number of virus. The dot blots were probed with β-galactosidase gene, and particle numbers were determined by control amounts of the β-galactosidase gene. Peak fractions containing virus were dialysed against PBS, 20% glycerol.

Example 23

Infection of Cells with Chimeric Virus

Forty-eight hours post-transfection, cell lysates were generated and tested for transduction into various target cells. A transducing titer of 2 $x10^6$ was generated. Various volumes of virus were added to 293, RT-2 rat glioma, U-87 glioma, as well as to two primary human glioblastoma cell lines in small volumes of medium. Virus was also added to UT7 megakaryoctye cells that had been incubated in the presence of erythropoeitin (EPO) for

several weeks. Exposure of UT7 cells to EPO is known to render these cells permissive for B19 infection.

Adenovirus was also added to the cells at an MOI of 5. Two hours after infection the virus was washed off and fresh medium was added. Twenty-four hours post infection the cells were washed with PBS, fixed in formaldehyde/gluteraldehyde, and stained with X-gal. Twelve to twenty-four hours later the number of blue cells was determined by counting ten fields.

Transduction was obtained in the glioma and primary human glioblastoma cells. Efficient transduction was not observed in 293 cells (a cell type typically infected with AAV). Interestingly, transduction was seen with the UT7 cells. These results suggest that the chimera has lost the native AAV tropism and has acquired the B19 tropism for erythroid cells. This virus is characterized to determine whether it has retained the antigenic properties associated with the AAV2 serotype.

The B19 globoside binding region (loop 4 between amino acids 399-406 of the Vp2 subunit; Brown *et al.* (1993) *Science* **262**:114) of this chimeric virus is deleted, modified or swapped out to reduce or completely eliminate the B19 tropism for erythroid cells.

Example 24

Characterization of B19/AAV Chimera

The results from **Example 23** indicate that a transducing chimeric virus was successfully generated. The chimeric virus was further evaluated for total particle yield and integrity. The remainder of the vector preparation was gradient purified, and the chimeric virus was analyzed by dot blot analysis to determine a particle titer of 1 $x10^8$ and EM analysis (*see* **Example 6**) to determine if a correct icosahedral structure was formed (**Figure 9**). From this analysis, it was confirmed that the chimeric virion that was generated retained the typical parvovirus structure and was stable to physical purification step such as sonication and $CsCl_2$ gradient centrifugation. This is an important observation since most parvovirus are heat stable (resistant up to 65

degrees), resistant to detergents (0.5% SDS) and can tolerate extreme pH changes (viable between pH of 2.0 - 11).

In addition, EM analysis yielded unexpected results (**Figure 9**). Virion particles of two different sizes were observed (a 23-28 nm particle, typical for wt AAV, and a 33-38 nm particle, never before identified). Further analysis suggested that the AAV 33-38 nm particle was formed by changing the triangulation number from T=1 to T=3, resulting in larger particles containing 180 copies of the major capsid component instead of 60. These surprising results indicate that a virion structure larger than wt AAV has been generated. This virion may have the potential for carrying larger than wt vector templates. The larger 33-38 nm particle will be useful in increasing packaging limits above the 6 kb range (the B19 25 nm particle packages 6 kb of DNA).

Example 25

Packaging Capacity of B19/AAV-2 Chimera

To quantitate the packaging capacity of the chimeric virus from **Example 21**, a series of vectors developed by Dong and coworkers, (1996) *Human Gene Therapy* **7**:2101, is utilized with genomes of progressively increased sizes having inserts between 745 and 1811 bases (for a maximum total genome size of 6.4 kb). Small-scale production of chimeric recombinant virus is used to assay packaging efficiency by testing the DNA content of the virus using Hirt assay, and by chloramphenicol acetyltransferase (CAT) reporter assay.

Example 26

Construction of Other B19/AAV Chimeras

Other chimeric B19/AAV capsids are generated as in **Example 21** (*e.g.*, swapping AAV Vp1 or Vp2 with B19 Vp1) and are characterized as described in **Examples 22-25** above. In particular, both B19 Vp1 and Vp2 are substituted into an AAV Vp1 chimera to generate a novel chimeric capsid containing AAV Vp1 and B19 Vp1 and Vp2.

These chimeras are assayed in 293 (typically infected by AAV) and erythroid cells (the cell type typically infected by B19) for transduction efficiency and are assayed for packaging recombinant AAV vectors with increasing sized inserts as described above.

If desired, the B19 globoside binding region (loop 4 of Vp2 between amino acids 399-406; Brown *et al.* (1993) *Science* **262**:114) of these vectors can be deleted, modified or swapped out to remove the B19 tropism.

Example 27

Loop Swaps Between AAV Serotypes

The capsid gene of AAV2, in the helper vector pACG2, was digested with the enzymes Asp718 and Bsi WI. Bsi WI has a unique site in the AAV2 genome at position 3254bp, and Asp718 digests the genome twice at 1906 and 4158bps (AAV2 sequence numbers). The capsid coding domain of AAV2 was partially digested with Asp718 and the full length (single cut) fragment was isolated. This fragment was then digested with Bsi WI and the 7272bp fragment isolated. This fragment removed the 904bp fragment the contains the coding region of the VP3 loop 2, 3, and 4 domains.

The capsid gene of AAV4 was digested with Asp718 and Bsi WI to completion and a 928 bp fragment from 3284 bp (BsiWI) to 4212bps (Asp718) was isolated (AAV4 sequence numbers). This AAV4 fragment codes for a region in VP3 that contains loops 2, 3 and 4. The 928bp AAV4 fragment and the 7272bp fragment from pACG2 were ligated and clones were identified.

These clones were used to make a chimeric virus that contained mostly AAV2 and part of the VP3 domain of AAV4. This virus did not infect HeLa cells as determined by blue stained cells (viral infected cells expressing the LacZ marker gene). However, like AAV4 these cells infected COS7 cells at a low titer of 1×10^5 transducing units/mL. These virions are not recognized by the AAV2 monoclonal antibody B1.

Chimeric virus was also made in which Vp3 Loops 2-4 from AAV2 were substituted into the homologous region of the AAV4 capsid.

The AAV3 capsid coding region containing the VP3 loops 2-4 domains were cloned into pACG2 in the same manner as described above for AAV2/4 loop swaps. These chimeric AAV2/3 virions bind heparin agarose and infect HeLa and 293 cells. Furthermore, these virions are recognized by the B1 monoclonal antibody.

Likewise, using the techniques taught above, Vp3 loops 2-4 from AAV5 are substituted for loops 2-4 of AAV2.

Furthermore, single loops (*e.g.*, loop 2, 3 or 4, or loops 2-3 or 3-4) are substituted from AAV3, 4 or 5 into AAV2 or vice versa.

These mutant viral vectors are characterized for virion formation, morphology, genome protection, heparin binding, and infectivity as described in **Example 4-7**.

A representative helper plasmid encoding a chimeric AAV2/3 capsid is given in **Appendix 5** (**SEQ ID:5**). This sequence contains the AAV2 rep coding sequences, most of the AAV2 capsid coding sequences, with the exception that loops 2-4 from the AAV2 Vp3 subunit were replaced with the corresponding region from AAV3, in a pBluescript backbone. The Rep 68/78 coding sequence starts at nu 251, and the Rep52/40 coding sequence starts at nu 923. The rep coding sequences end at nu 2114 for Rep78/52 and at nu 2180 for Rep68/40. The cap coding region starts at nu 2133 and ends at nu 4342 (Vp1 start at nu 2133, Vp2 start at nu 2544, Vp3 start at nu 2739).

Briefly, both AAV2 (pACG2) and AAV3 helper plasmids were digested with Bsi WI and Asp 718. This removes a 904 bp fragment in the AAV2 genome from nu 3255 to 4159. In the AAV3 genome, the same digestion removed 907 bp from nu 3261-4168. This 904 bp fragment was ligated into the deleted AAV2 helper to result in the helper given in **SEQ ID NO:5** (AAV3 sequences at nu 3184-4092 of the plasmid).

Example 28

Hybrid Viruses

Primers were made to create a unique Hind III site in the AAV4 rep

gene that overlapped the Hind III site in AAV2. In addition, at the 3' end of the rep coding sequence, a unique Not I site was created 3' of the polyadenylation site. A virus purchased from American Type Culture Collection (ATCC) as the template for the PCR.

The 5' portion of the AAV2 rep gene from the Xba I site to the Hind III site was subcloned into pBluescript. The Hind III-Not I PCR digestion product was then cloned into the pBluescript containing the 5' rep gene digested with Not I and Hind III.

Primers:

AAV4 3' Not I primer (**SEQ ID NO:58**):

5' -AAG CGC CGC GGC CGC TGC TTA TGT ACG CA- 3'

AAV4 5' Hind III primer (**SEQ ID NO:59**):

5' -GAC GCG GAA GCT TCG GTG GAC TAC GCG- 3'

This cloning strategy resulted in a helper plasmid that is a hybrid for AAV2 and AAV4 rep genes and contains the AAV4 cap genes. This helper contains the AAV2 rep gene up to the Hind III site and from this past the polyadenylation site the sequences are derived from AAV4.

This virus packaged a recombinant AAV2 genome with AAV2 ITRs. This hybrid AAV2/4 virus exhibits the binding characteristics of AAV4, *e.g.*, it does not bind HS and transduces AAV4 target cells that are not typically permissive to AAV2 transduction.

The hybrid AAV 2/4 helper plasmid is as given in **Appendix 1** (**SEQ ID NO:1**). This sequence encodes the AAV2 rep genes and AAV4 capsid in a pBluescript backbone. The Rep 68/78 coding sequence starts at nu 251, and the Rep52/40 coding sequence starts at nu 923. The rep coding sequences end at nu 2120 for Rep78/52 and at nu 2183 for Rep68/40. The cap coding region starts at nu 2123 and ends at nu 4341 (Vp1 start at nu 2123, Vp2 start at nu 2547, Vp3 start at nu 2727).

Using the same techniques, a hybrid AAV2/3 virus in which a

recombinant AAV2 genome (with AAV2 ITRs) is packaged. The resulting hybrid virus is viable and efficiently transduces AAV3 permissive cells.

In addition, in contrast to a recent report (Chiorini et al., 1999) *J. Virology* **73**:1309), the techniques described above have been used to produce a hybrid AAV2/5 virus in which a recombinant AAV2 genome (with AAV2 ITRs) is packaged within a AAV Type 5 capsid. This virus is packaged relatively inefficiently, but the resulting particles demonstrated transduction of cells.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims and equivalents thereof.

That Which is Claimed is:

1. A hybrid virus particle comprising:

a parvovirus capsid; and

an AAV genome packaged within said parvovirus capsid, subject to the proviso that if said parvovirus capsid is an AAV capsid, the serotypes of said AAV capsid and said AAV genome are different.

2. The hybrid virus particle of Claim 1, wherein said AAV genome comprises at least one AAV inverted terminal repeat.

3. The hybrid virus particle of Claim 1, wherein said AAV genome is a recombinant AAV genome comprising at least one heterologous nucleic acid sequence.

4. The hybrid virus particle of Claim 1, wherein said parvovirus capsid is an autonomous parvovirus capsid.

5. The hybrid virus particle of Claim 1, wherein said parvovirus capsid is a B19 capsid.

6. The hybrid virus particle of Claim 3, wherein said AAV genome is an AAV serotype-2 genome.

7. The hybrid virus particle of Claim 1, wherein said parvovirus capsid is an AAV capsid.

8. The hybrid virus particle of Claim 7, wherein:

said AAV genome is of a serotype selected from the group consisting of AAV serotypes 1, 2, 3, 4, 5 and 6; and

said AAV capsid is of a serotype selected from the group consisting of AAV serotypes 1, 2, 3, 4, 5 and 6.

9. The hybrid virus particle of Claim 8 selected from the group consisting of:

(a) a hybrid virus particle comprising an AAV serotype-3 capsid and an AAV serotype-2 genome,

(b) a hybrid virus particle comprising an AAV serotype-4 capsid and an AAV serotype-2 genome, and

(c) a hybrid virus particle comprising an AAV serotype-5 capsid and an AAV serotype-2 genome.

10. The hybrid virus particle of Claim 1, wherein all of the AAV cap genes and all of the AAV rep genes are deleted from said AAV genome.

11. The hybrid virus particle of Claim 2 comprising two AAV inverted terminal repeats that flank said at least one heterologous nucleic acid sequence.

12. The hybrid virus particle of Claim 3, wherein said at least one heterologous nucleic acid sequence encodes a protein or peptide.

13. The hybrid virus particle of Claim 12, wherein said protein or peptide is a therapeutic protein or peptide.

14. The hybrid virus particle of Claim 12, wherein said protein or peptide is an immunogenic protein or peptide.

15. The hybrid virus particle of Claim 3, wherein said at least one heterologous nucleic acid sequence encodes an untranslated RNA.

16. A pharmaceutical formulation comprising the hybrid virus particle of Claim 1 in a pharmaceutically-acceptable carrier.

17. An isolated nucleic acid encoding the hybrid virus capsid of Claim 1, wherein said isolated nucleic acid comprises parvovirus cap genes and adeno-associated virus (AAV) rep genes, subject to the proviso that if said parvovirus cap genes are AAV cap genes, the serotypes of said AAV cap genes and said AAV rep genes are different.

18. The isolated nucleic acid of Claim 17, wherein said parvovirus cap genes are operably associated with an authentic parvovirus promoter.

19. The isolated nucleic acid of Claim 17, wherein said parvovirus cap genes are B19 cap genes.

20. The isolated nucleic acid of Claim 19, wherein said AAV rep genes are AAV serotype-2 rep genes.

21. The isolated nucleic acid of Claim 17, wherein said AAV rep genes encode at least one temperature-sensitive AAV Rep protein.

22. The isolated nucleic acid of Claim 17, wherein said cap genes are AAV cap genes.

23. The isolated nucleic acid of Claim 22, wherein said AAV cap genes are operably associated with an authentic AAV promoter.

24. The isolated nucleic acid of Claim 23, wherein said authentic AAV promoter is an AAV p40 promoter.

25. The isolated nucleic acid of Claim 22, wherein:

said AAV cap genes are of a serotype selected from the group consisting of AAV serotypes 1, 2, 3, 4, 5 and 6; and

said AAV rep genes are of a serotype selected from the group consisting of AAV serotypes 1, 2, 3, 4, 5 and 6.

26. The isolated nucleic acid of Claim 25 selected from the group consisting of:

(a) a vector comprising AAV serotype-3 cap genes and AAV serotype-2 rep genes,

(b) a vector comprising AAV serotype-4 cap genes and AAV serotype-2 rep genes, and

(c) a vector comprising AAV serotype-5 cap genes and AAV serotype-2 rep genes.

27. A vector comprising the isolated nucleic acid of Claim 17.

28. The vector of Claim 27, wherein said vector is selected from the group consisting of plasmids, naked DNA vectors, bacterial artificial chromosomes, yeast artificial chromosomes, and viral vectors.

29. The vector of Claim 28, wherein said vector is a plasmid.

30. A cell comprising the vector of Claim 29.

31. The cell of Claim 30, wherein said cell is selected from the group consisting of bacterial, protozoan, yeast, fungus, plant, and animal cells.

32. A cell comprising a vector comprising:
parvovirus cap genes,
adeno-associated virus (AAV) rep genes, and
an AAV genome,
subject to the proviso that if said parvovirus cap genes are AAV cap genes, said AAV genome is of a different AAV serotype than said cap genes.

33. The cell of Claim 32, wherein said cell is a mammalian cell.

34. A cell comprising parvovirus cap genes and adeno-associated virus (AAV) rep genes stably integrated into the genome of the packaging cell, subject to the proviso that if said parvovirus cap genes are AAV cap genes, the serotypes of said AAV cap genes and said AAV rep genes are different.

35. The cell of Claim 34 further comprising an AAV genome comprising, subject to the proviso that if said parvovirus cap genes are AAV cap genes, the serotypes of said AAV cap genes and said AAV genome are different.

36. A method of producing a hybrid virus particle, comprising:

providing a cell with adeno-associated virus (AAV) rep genes, parvovirus cap genes, an AAV genome, and helper functions for generating a productive AAV infection; subject to the proviso that if the parvovirus cap genes are AAV cap genes, the serotypes of the AAV cap genes and the AAV genome are different, and

allowing assembly of the hybrid virus particles.

37. The method of Claim 36, further comprising collecting the hybrid virus particles.

38. The method of Claim 36, wherein the AAV genome comprises at least one AAV inverted terminal repeat.

39. The method of Claim 36, wherein the AAV genome is a recombinant AAV genome comprising at least one heterologous nucleic acid sequence.

40. The method of Claim 36, wherein the parvovirus cap genes and AAV rep genes are provided by one or more transcomplementing packaging vectors.

41. The method of Claim 36, wherein the parvovirus cap genes and AAV rep genes are provided by a plasmid.

42. The method of Claim 36, wherein the parvovirus cap genes and AAV rep genes are provided by an adenovirus vector.

43. The method of Claim 36, wherein the AAV rep genes encode at least one temperature-sensitive AAV Rep protein.

44. The method of Claim 36, wherein the parvovirus cap genes and AAV rep genes are stably integrated into the genome of the cell.

45. The method of Claim 36, wherein the parvovirus cap genes are AAV cap genes.

46. A hybrid virus particle produced by the method of Claim 36.

47. A method of delivering a nucleic acid sequence to a cell, comprising:

introducing into a cell a hybrid virus particle comprising a parvovirus capsid and an adeno-associated virus (AAV) genome packaged within the capsid, the AAV genome, subject to the proviso that if the parvovirus capsid is an AAV capsid, the serotypes of the AAV capsid and the AAV genome are different.

48. The method of Claim 47, wherein the AAV genome comprises at least one AAV inverted terminal repeat.

49. The method of Claim 47, wherein the AAV genome is a recombinant AAV genome comprising at least one heterologous nucleic acid sequence.

50. The method of Claim 49, wherein the heterologous nucleic acid sequence is expressed in the cell.

51. The method of Claim 47, wherein the parvovirus capsid is a B19 capsid.

52. The method of Claim 49, wherein the at least one heterologous nucleic acid sequence encodes a protein or peptide.

53. The method of Claim 52, wherein the protein or peptide is a therapeutic protein or peptide.

54. The method of Claim 50, wherein the protein or peptide is an immunogenic protein or peptide.

55. The method of Claim 49, wherein the heterologous nucleic acid sequence encodes an untranslated RNA.

56. The method of Claim 47, wherein the cell is selected from the group consisting of a neural cell, lung cell, retinal cell, epithelial cell, muscle cell, pancreatic cell, hepatic cell, myocardial cell, bone cell, spleen cell, keratinocyte, fibroblast, endothelial cell, prostate cell, germ cell, progenitor cell, and a stem cell.

57. The method of Claim 47, wherein the parvovirus capsid is an AAV capsid.

58. The method of Claim 57, wherein:

the AAV genome is of a serotype selected from the group consisting of AAV serotypes 1, 2, 3, 4, 5 and 6; and

the AAV capsid is of a serotype selected from the group consisting of AAV serotypes 1, 2, 3, 4, 5 and 6.

59. The method of Claim 58, wherein the hybrid virus particle is selected from the group consisting of:

(a) a hybrid virus particle comprising an AAV serotype-3 capsid and an AAV serotype-2 genome,

(b) a hybrid virus particle comprising an AAV serotype-4 capsid and an AAV serotype-2 genome, and

(c) a hybrid virus particle comprising an AAV serotype-5 capsid and an AAV serotype-2 genome.

60. A method of administering a nucleic acid to a subject comprising administering the cell of Claim 47 to a subject.

61. A method of administering a nucleic acid sequence to a subject, comprising administering to a subject a hybrid virus particle comprising a parvovirus capsid and an adeno-associated virus (AAV) genome packaged within the capsid, subject to the proviso that if the parvovirus capsid is from AAV, the serotypes of the AAV capsid and the AAV genome are different.

62. The method of Claim 61, wherein the AAV genome comprises at least one AAV inverted terminal repeat.

63. The method of Claim 61, wherein the AAV genome is a recombinant AAV genome comprising at least one heterologous nucleic acid sequence.

64. The method of Claim 61, wherein the subject is selected from the group consisting of avian subjects and mammalian subjects.

65. The method of Claim 62, wherein the subject is a human subject.

66. The method of Claim 62, wherein the subject is seropositive for the serotype of the AAV genome.

67. The method of Claim 62, wherein the hybrid virus particle is administered by a route selected from the group consisting of oral, rectal, transmucosal, transdermal, inhalation, intravenous, subcutaneous, intradermal, intracranial, intramuscular, and intraarticular administration.

68. The method of Claim 62, wherein the hybrid virus particle is administered to the liver of the subject.

69. The method of Claim 68, wherein the hybrid virus particle is administered to the liver by a route selected from the group consisting of intravenous administration, intraportal administration, intrabiliary administration, intra-arterial administration, and direct injection into the liver parenchyma.

70. The method of Claim 63, wherein the at least one heterologous nucleic acid sequence encodes a protein or peptide.

71. The method of Claim 62, wherein the parvovirus capsid is an AAV capsid.

72. A chimeric parvovirus capsid comprising at least one capsid region from an adeno-associated virus (AAV) and at least one capsid region from a B19 virus.

73. A chimeric parvovirus comprising the capsid of Claim 72 and an AAV genome.

74. The chimeric parvovirus of Claim 73, wherein said parvovirus packages larger than wild-type AAV genomes.

75. The chimeric parvovirus of Claim 73, wherein said parvovirus is about 33-38 nanometers in diameter.

76. The chimeric parvovirus of Claim 73 comprising an AAV capsid comprising a capsid B19 subunit.

77. The chimeric parvovirus of Claim 76, wherein an AAV capsid subunit is replaced by a B19 capsid subunit.

78. The chimeric parvovirus of Claim 77, wherein the Vp3 subunit of the AAV capsid is replaced by the Vp2 subunit of B19.

79. A chimeric parvovirus capsid protein comprising at least one capsid region from a different parvovirus.

80. The chimeric parvovirus capsid protein of Claim 79, wherein an antigenic property related to the serotype of said parvovirus capsid protein is reduced as compared with the wild-type parvovirus capsid protein.

81. The chimeric parvovirus capsid protein of Claim 79, wherein said capsid protein is an adeno-associated virus (AAV) capsid protein.

82. A chimeric virus capsid comprising the chimeric parvovirus capsid protein of Claim 79.

83. A chimeric virus particle comprising:

(a) a chimeric parvovirus capsid of Claim 82; and

(b) an AAV genome packaged within the chimeric parvovirus capsid.

84. The chimeric virus particle of Claim 83, wherein said AAV genome comprises at least one AAV inverted terminal repeat.

85. The chimeric virus particle of Claim 83, wherein said AAV genome comprises at least one heterologous nucleic acid sequence.

86. The chimeric virus particle of Claim 83, wherein said capsid region from said different parvovirus is inserted into said parvovirus capsid.

87. The chimeric virus particle of Claim 83, wherein said at least one capsid region from said different parvovirus replaces a region within said parvovirus capsid.

88. The chimeric virus particle of Claim 87, wherein said at least one capsid region from said different parvovirus replaces a homologous region within said parvovirus capsid.

89. The chimeric virus particle of Claim 83, wherein said at least one capsid region from said different parvovirus is a loop region of the major capsid subunit.

90. The chimeric virus particle of Claim 89, wherein said loop region replaces a loop region in the major subunit of said parvovirus capsid.

91. The chimeric virus particle of Claim 83, wherein said at least one capsid region from said different parvovirus replaces a capsid subunit in said parvovirus capsid.

92. The chimeric virus particle of Claim 83, wherein said parvovirus capsid is an autonomous parvovirus capsid.

93. The chimeric virus particle of Claim 83, wherein said parvovirus capsid is an adeno-associated virus (AAV) capsid.

94. The chimeric virus particle of Claim 93, wherein an antigenic property related to the serotype of said AAV capsid is reduced as compared with the wild-type AAV capsid.

95. The chimeric virus particle of Claim 93, wherein said AAV capsid is a serotype-2 AAV capsid.

96. The chimeric virus particle of Claim 93, wherein said AAV genome is of the same serotype as said AAV capsid.

97. The chimeric virus particle of Claim 93, wherein said AAV genome is a serotype-2 AAV genome.

98. The chimeric virus particle of Claim 83, wherein said AAV genome is a serotype-2 AAV genome.

99. The chimeric virus particle of Claim 83, wherein said different parvovirus is an AAV.

100. The chimeric virus particle of Claim 83, wherein said different parvovirus is an autonomous parvovirus.

101. A pharmaceutical formulation comprising said chimeric virus particle of Claim 83 in a pharmaceutically-acceptable carrier.

102. An isolated nucleic acid encoding the chimeric virus capsid protein of Claim 79.

103. The isolated nucleic acid of Claim 102, wherein said at least one capsid region is inserted into said chimeric capsid protein.

104. The isolated nucleic acid of Claim 102, wherein said at least one capsid region replaces sequences within said chimeric capsid protein.

105. The isolated nucleic acid of Claim 102, wherein said chimeric parvovirus capsid protein is a chimeric AAV capsid protein.

106. The isolated nucleic acid of Claim 105, wherein said isolated nucleic acid comprises the AAV cap genes and the AAV rep genes.

107. A vector comprising the isolated nucleic acid of Claim 102.

108. A cell comprising the vector of Claim 107.

109. The cell of Claim 108 further comprising an adeno-associated virus (AAV) genome.

110. A cell comprising the isolated nucleic acid of Claim 102 stably integrated into the genome of the cell.

111. The cell of Claim 110 further comprising an adeno-associated virus (AAV) genome.

112. A method of producing a chimeric virus particle, comprising:

providing a cell with parvovirus cap genes, rep genes from an adeno-associated virus (AAV), an AAV genome, and helper functions for generating a productive AAV infection; wherein the cap genes comprise at

least one nucleic acid sequence from the cap genes of a different parvovirus; and

allowing assembly of the chimeric virus particles.

113. The method of Claim 112, further comprising collecting the chimeric virus particles.

114. The method of Claim 112, wherein the AAV genome comprises at least one AAV inverted terminal repeat.

115. The method of Claim 112, wherein the AAV genome is a recombinant AAV genome comprising at least one heterologous nucleic acid sequence.

116. The method of Claim 112, wherein the at least one nucleic acid sequence is inserted into the parvovirus cap genes.

117. The method of Claim 112, wherein the at least one nucleic acid sequence replaces sequences within the parvovirus cap genes.

118. The method of Claim 112, wherein the parvovirus cap genes and AAV rep genes are provided by one or more transcomplementing packaging vectors.

119. The method of Claim 112, wherein the parvovirus cap genes and AAV rep genes are provided by a plasmid.

120. The method of Claim 112, wherein the parvovirus cap genes and AAV rep genes are stably integrated into the genome of the cell.

121. The method of Claim 112, wherein the parvovirus is an AAV.

122. The method of Claim 121, wherein the AAV cap genes and AAV rep genes are of the same serotype.

123. The method of Claim 112, wherein the AAV rep genes are serotype-2 AAV rep genes.

124. A chimeric virus particle produced by the method of Claim 112.

125. A method of delivering a nucleic acid sequence to a cell, comprising:

introducing into a cell a chimeric virus particle comprising a parvovirus capsid and an adeno-associated virus (AAV) genome packaged within the capsid,

wherein the parvovirus capsid comprises at least one capsid region from a different parvovirus.

126. The method of Claim 125, wherein the AAV genome comprises and at least one AAV inverted terminal repeat.

127. The method of Claim 125, wherein the AAV genome is a recombinant AAV genome comprising at least one heterologous nucleic acid sequence.

128. The method of Claim 127, wherein the at least one heterologous nucleic acid sequence encodes a protein or peptide.

129. The method of Claim 125, wherein the cell is selected from the group consisting of a neural cell, lung cell, retinal cell, epithelial cell, muscle cell, pancreatic cell, hepatic cell, myocardial cell, bone cell, spleen cell, keratinocyte, fibroblast, endothelial cell, prostate cell, germ cell, progenitor cell, and a stem cell.

130. The method of Claim 125, wherein the parvovirus capsid is an AAV capsid.

131. The method of Claim 130, wherein the at least one capsid region is from a B19 virus.

132. The method of Claim 131, wherein the Vp3 subunit of the AAV capsid is replaced by the Vp2 subunit of B19.

133. The method of Claim 125, wherein said AAV genome is a serotype-2 AAV genome.

134. A method of administering a nucleic acid to a subject comprising administering the cell of Claim 125 to a subject.

135. A method of administering a nucleic acid sequence to a subject, comprising administering to a subject a chimeric virus particle comprising a parvovirus capsid and an adeno-associated virus (AAV) genome packaged within the capsid,

wherein the parvovirus capsid comprises at least one capsid region from a different parvovirus.

136. The method of Claim 135, wherein the AAV genome comprises at least one AAV inverted terminal repeat.

137. The method of Claim 135, wherein the AAV genome comprises at least one heterologous nucleic acid sequence.

138. The method of Claim 135, wherein the subject is selected from the group consisting of avian subjects and mammalian subjects.

139. The method of Claim 136, wherein the subject is a human subject.

140. The method of Claim 135, wherein the subject is seropositive for the serotype of the AAV genome.

141. The method of Claim 135, wherein the chimeric virus particle is administered by a route selected from the group consisting of oral, rectal, transmucosal, transdermal, inhalation, intravenous, subcutaneous, intradermal, intracranial, intramuscular, and intraarticular administration.

142. The method of Claim 135, wherein the chimeric virus particle is administered to the liver of the subject.

143. The method of Claim 135, wherein the parvovirus capsid is an AAV capsid.

144. The method of Claim 135, wherein the AAV genome is a serotype-2 AAV genome.

145. The method of Claim 135, wherein an antigenic property related to the serotype of said AAV capsid is reduced as compared with the wild-type AAV capsid.

146. A targeted parvovirus capsid protein comprising at least one exogenous targeting sequence, wherein said at least one exogenous targeting sequence confers an altered tropism to a virus particle comprising said targeted parvovirus capsid protein.

147. The targeted parvovirus capsid protein of Claim 146, wherein said parvovirus capsid protein is an autonomous parvovirus capsid protein.

148. The targeted parvovirus capsid protein of Claim 146, wherein said parvovirus capsid protein is an adeno-associated virus (AAV) capsid protein.

149. The targeted parvovirus capsid protein of Claim 146, wherein said at least one exogenous targeting sequence is a capsid sequence from an autonomous parvovirus.

150. The targeted parvovirus capsid protein of Claim 146, wherein said at least one exogenous targeting sequence is a capsid sequence from an AAV.

151. The targeted parvovirus capsid protein of Claim 146, wherein said at least one exogenous targeting sequence encodes a protein or peptide that binds to a cell-surface receptor.

152. The targeted parvovirus capsid protein of Claim 146, wherein said at least one exogenous targeting sequence encodes a receptor ligand.

153. The targeted parvovirus capsid protein of Claim 146, wherein a tropism of a virus particle comprising said parvovirus capsid protein is reduced or eliminated.

154. The targeted parvovirus capsid protein of Claim 146, wherein a tropism of a virus particle comprising said targeted parvovirus capsid protein is enhanced.

155. The targeted parvovirus capsid protein of Claim 146, wherein a virus particle comprising said targeted parvovirus capsid protein acquires a new tropism.

156. A virus capsid comprising the targeted parvovirus capsid protein of Claim 146.

157. The virus capsid of Claim 156, wherein said virus capsid is a parvovirus capsid.

158. The virus capsid of Claim 157, wherein said parvovirus capsid is an adeno-associated virus capsid.

159. A targeted virus particle comprising a parvovirus capsid comprising:

the virus capsid of Claim 156, wherein said virus capsid is a parvovirus capsid; and

an adeno-associated virus (AAV) genome.

160. The targeted virus particle of Claim 159, wherein said AAV genome comprises at least one AAV inverted terminal repeat.

161. The targeted virus particle of Claim 159, wherein said AAV genome is a recombinant AAV genome comprising at least one heterologous nucleic acid sequence.

162. The targeted virus particle of Claim 159, wherein said AAV genome is a serotype-2 AAV genome.

163. The targeted virus particle of Claim 159, wherein said parvovirus is an autonomous parvovirus.

164. The targeted virus particle of Claim 159, wherein said parvovirus is an AAV.

165. The targeted virus particle of Claim 159, wherein said at least one exogenous targeting sequence is a capsid sequence from an autonomous parvovirus.

166. The targeted virus particle of Claim 159, wherein said at least one exogenous targeting sequence is a capsid sequence from an AAV.

167. The targeted virus particle of Claim 159, wherein said at least one exogenous targeting sequence encodes a protein or peptide that binds to a cell-surface receptor.

168. The targeted virus particle of Claim 159, wherein said at least one exogenous targeting sequence encodes a receptor ligand.

169. The targeted virus particle of Claim 159, wherein said at least one exogenous targeting sequence encodes an antibody or a fragment thereof.

170. The targeted virus particle of Claim 159, wherein said parvovirus capsid and said AAV genome are of the same serotype.

171. The targeted virus particle of Claim 159, wherein the AAV cap genes and AAV rep genes are deleted from said AAV genome.

172. The targeted virus particle of Claim 159, wherein a tropism of said parvovirus is reduced or eliminated.

173. The targeted virus particle of Claim 159, wherein a tropism of said parvovirus is enhanced.

174. The targeted virus particle of Claim 159, wherein said parvovirus acquires a new tropism.

175. The targeted virus particle of Claim 159, wherein a tropism of said parvovirus is reduced or eliminated and the parvovirus acquires a new tropism.

176. The targeted virus particle of Claim 159, wherein a tropism of said parvovirus is reduced or eliminated and another tropism of said parvovirus is enhanced.

177. The targeted virus particle of Claim 159, wherein said at least one exogenous targeting sequence encodes bradykinin or a fragment thereof.

178. The targeted virus particle of Claim 164, wherein a nucleotide sequence encoding said at least one exogenous targeting sequence is inserted or substituted into the nucleotide sequence encoding the AAV capsid at a position selected from the group consisting of nucleotide 2285, 2356, 2364, 2416, 2591, 2634, 2690, 2747, 2944, 3317, 3391, 3561, 3595, 3753, 3761, 3766, 3789, 3858, 3960, 3961, 3987, 4046, 4047 and 4160 of the AAV serotype 2 genome, or the corresponding region of AAV of other serotypes, wherein the inserted or substituted sequence begins at the nucleotide following the indicated position.

179. The targeted virus particle of Claim 164, wherein an exogenous targeting sequence is inserted or substituted into the AAV capsid at a position selected from the group consisting of amino acid 28, 51, 54, 71, 130, 144, 163, 182, 247, 372, 396, 452, 464, 520, 521, 517, 529, 552, 586, 595, 615, and 653 of the AAV serotype 2 Vp1 capsid subunit and the corresponding position in the Vp2 and Vp3 capsid subunits, wherein the inserted or substituted sequence begins at the amino acid following the indicated position.

180. The targeted virus particle of Claim 179, wherein said virus particle consists of two exogenous targeting sequences.

181. A pharmaceutical formulation comprising the targeted virus particle of Claim 159 in a pharmaceutically-acceptable carrier.

182. An isolated nucleic acid encoding the targeted parvovirus capsid protein of Claim 146.

183. The isolated nucleic acid of Claim 182, wherein said parvovirus capsid protein is an autonomous parvovirus capsid protein.

184. The isolated nucleic acid of Claim 182, wherein said parvovirus capsid protein is an adeno-associated virus (AAV) capsid protein.

185. The isolated nucleic acid of Claim 182, wherein said isolated nucleic acid encodes the parvovirus cap genes.

186. The isolated nucleic acid of Claim 185 further comprising the AAV rep genes.

187. A vector comprising the isolated nucleic acid of Claim 182.

188. The vector of Claim 187, wherein said vector is selected from the group consisting of plasmids, naked DNA vectors, bacterial artificial chromosomes, yeast artificial chromosomes, and viral vectors.

189. A cell comprising the vector of Claim 188.

190. The cell of Claim 189 further comprising an adeno-associated virus (AAV) genome.

191. A cell comprising the isolated nucleic acid of Claim 182 stably integrated into the genome of said cell.

192. The cell of Claim 191 further comprising an adeno-associated virus (AAV) genome.

193. A method of producing a virus particle, comprising:

providing a cell with parvovirus cap genes, rep genes from an adeno-associated virus (AAV), an AAV genome, and helper functions for generating a productive AAV infection; wherein said cap genes comprise at least one nucleic acid sequence encoding an exogenous targeting sequence; and

allowing assembly of the virus particles containing said at least one exogenous amino acid sequence, wherein said at least one exogenous targeting sequence confers an altered tropism upon the virus particles.

194. The method of Claim 193, further comprising collecting the virus particles.

195. The method of Claim 193, wherein the AAV genome comprises at least one AAV inverted terminal repeat.

196. The method of Claim 193, wherein the AAV genome is a recombinant AAV genome comprising at least one heterologous nucleic acid sequence.

197. The method of Claim 193, wherein a virus stock is producing with a titer of at least about 10^5 transducing units/ml.

198. The method of Claim 193, wherein a virus stock is produced with a titer of at least about 1 transducing unit/cell.

199. The method of Claim 193, wherein the at least one nucleic acid sequence is inserted into the parvovirus cap genes.

200. The method of Claim 193, wherein the at least one exogenous nucleic acid sequence replaces sequences within the parvovirus cap genes.

201. The method of Claim 193, wherein the parvovirus cap genes and AAV rep genes are provided by one or more transcomplementing packaging vectors.

202. The method of Claim 201, wherein the parvovirus cap genes and AAV rep genes are provided by a plasmid.

203. The method of Claim 193, wherein the parvovirus cap genes and AAV rep genes are stably integrated into the genome of the cell.

204. The method of Claim 193, wherein the parvovirus cap genes are AAV cap genes.

205. A virus particle produced by the method of Claim 193.

206. A method of delivering a nucleic acid sequence to a cell, comprising:

introducing into a cell a targeted virus particle comprising a parvovirus capsid and an adeno-associated virus (AAV) genome packaged within the capsid, the parvovirus capsid comprising at least one exogenous targeting sequence, wherein the at least one exogenous targeting sequence confers an altered tropism to the targeted virus particle.

207. The method of Claim 206, wherein the AAV genome comprises at least one AAV inverted terminal repeat.

208. The method of Claim 206, wherein the AAV genome is a recombinant AAV genome comprising at least one heterologous nucleic acid sequence.

209. The method of Claim 206, wherein the cell is contacted with a composition comprising at least about 10^6 transducing units of the targeted virus particle.

210. The method of Claim 206, wherein the at least one heterologous nucleic acid sequence encodes a protein or peptide.

211. The method of Claim 206, wherein the cell is selected from the group consisting of a neural cell, lung cell, retinal cell, epithelial cell, muscle cell, pancreatic cell, hepatic cell, myocardial cell, bone cell, spleen cell, keratinocyte, fibroblast, endothelial cell, prostate cell, germ cell, prostate cell, progenitor cell, and a stem cell.

212. The method of Claim 206, wherein the parvovirus capsid is an AAV capsid.

213. The method of Claim 206, wherein the tropism of the parvovirus for the cell is enhanced.

214. The method of Claim 206, wherein the parvovirus essentially does not infect or transduce the cell in the absence of the exogenous targeting sequence.

215. The method of Claim 206, wherein the at least one exogenous targeting sequence encodes bradykinin or a fragment thereof.

216. The method of Claim 206, wherein the AAV genome is a serotype-2 AAV genome.

217. A method of administering a nucleic acid to a subject comprising administering the cell of Claim 206 to the subject.

218. A method of administering a nucleic acid sequence to a subject, comprising administering to a subject a targeted virus particle comprising a parvovirus capsid and an adeno-associated virus (AAV) genome packaged within the capsid, the parvovirus capsid comprising at least one exogenous targeting sequence, wherein the exogenous targeting sequence confers an altered tropism to the targeted virus particle.

219. The method of Claim 218, wherein AAV genome comprises at least one AAV inverted terminal repeat.

220. The method of Claim 218, wherein said AAV genome is a recombinant AAV genome comprising at least one heterologous nucleic acid sequence.

221. The method of Claim 218, wherein the subject is administered a composition comprising at least about 10^6 transducing units of the targeted virus particle.

222. The method of Claim 218, wherein the subject is selected from the group consisting of avian subjects and mammalian subjects.

223. The method of Claim 222, wherein the subject is a human subject.

224. The method of Claim 218, wherein the virus particle is administered by a route selected from the group consisting of oral, rectal, transmucosal, transdermal, inhalation, intravenous, subcutaneous, intradermal, intracranial, intramuscular, and intraarticular administration.

225. The method of Claim 218, wherein the virus particle is administered to the liver of the subject.

226. The method of Claim 218, wherein the parvovirus capsid is an AAV capsid.

227. The method of Claim 218, wherein the tropism of the parvovirus for a cell is enhanced.

228. The method of Claim 218, wherein the parvovirus capsid acquires a new tropism.

229. The method of Claim 218, wherein the AAV genome is a serotype-2 AAV genome.

230. A virus particle comprising a parvovirus capsid comprising at least one exogenous amino acid sequence, wherein said at least one exogenous amino acid sequence comprises a sequence that facilitates purification of the virus particle.

231. The virus particle of Claim 230, wherein said parvovirus is an adeno-associated virus (AAV).

232. An adeno-associated virus (AAV) capsid comprising at least one exogenous amino acid sequence is inserted or substituted into the AAV capsid at a position selected from the group consisting of amino acid 28, 51, 54, 71, 130, 144, 163, 182, 247, 372, 396, 452, 464, 520, 521, 517, 529, 552, 586, 595, 615, and 653 of the AAV serotype 2 Vp1 capsid subunit and the corresponding position in the Vp2 and Vp3 capsid subunits, wherein the inserted or substituted sequence begins at the amino acid following the indicated position.

233. The AAV capsid of Claim 232, wherein the exogenous amino acid sequence encodes an immunogenic peptide or protein.

234. The AAV capsid of Claim 232 covalently linked bound to, or encapsidating a compound selected from the group consisting of a DNA molecule, an RNA molecule, a protein, a peptide, a carbohydrate, a lipid, and a small organic molecule.

5

235. An AAV particle comprising the capsid of Claim 232.

FIG. 1

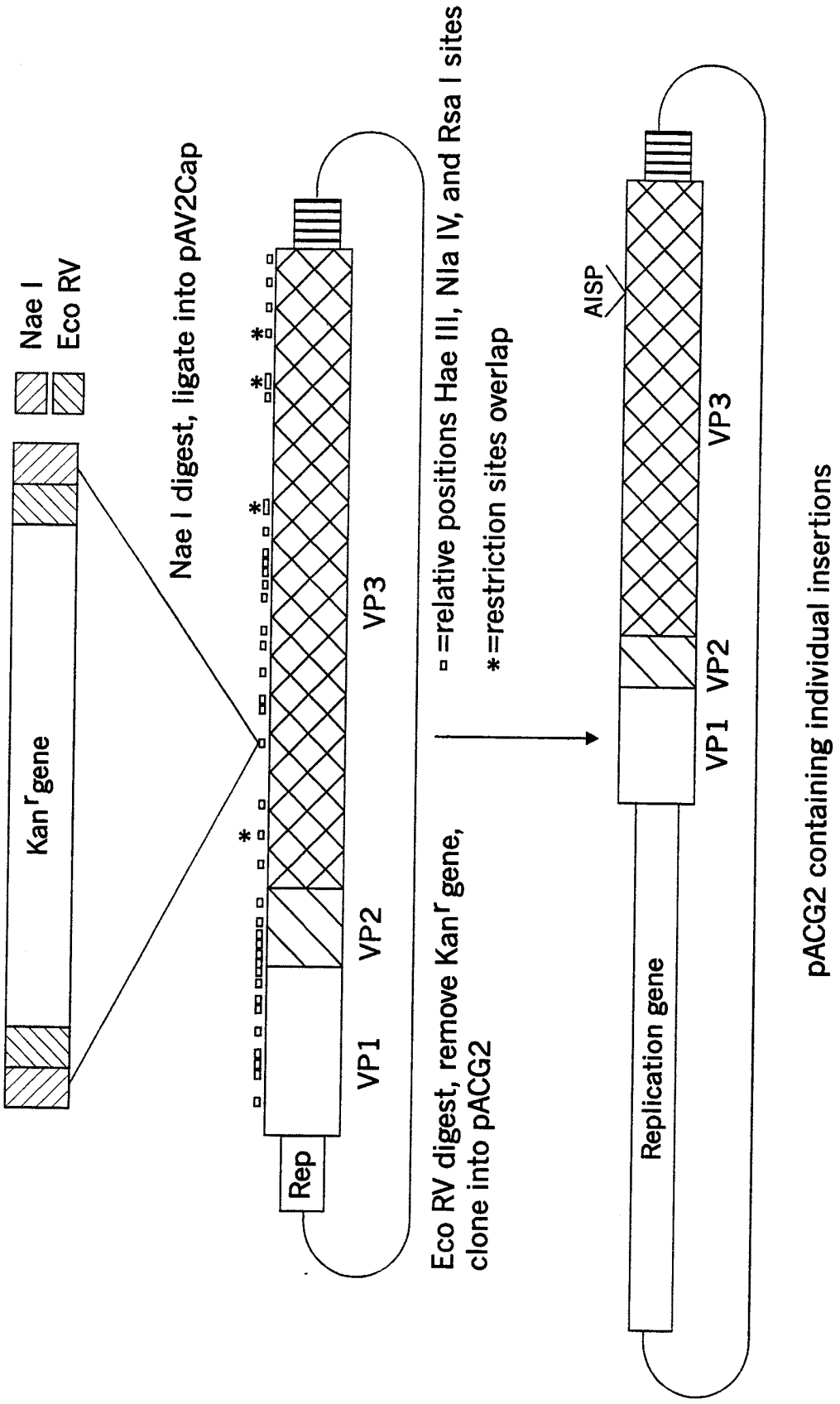

Nae I
Eco RV
Kan^r gene
Nae I digest, ligate into pAV2Cap
Rep
VP1
VP2
VP3
Eco RV digest, remove Kan^r gene, clone into pACG2
▫=relative positions Hae III, Nla IV, and Rsa I sites
*=restriction sites overlap
AISP
Replication gene
pACG2 containing individual insertions

FIG. 2

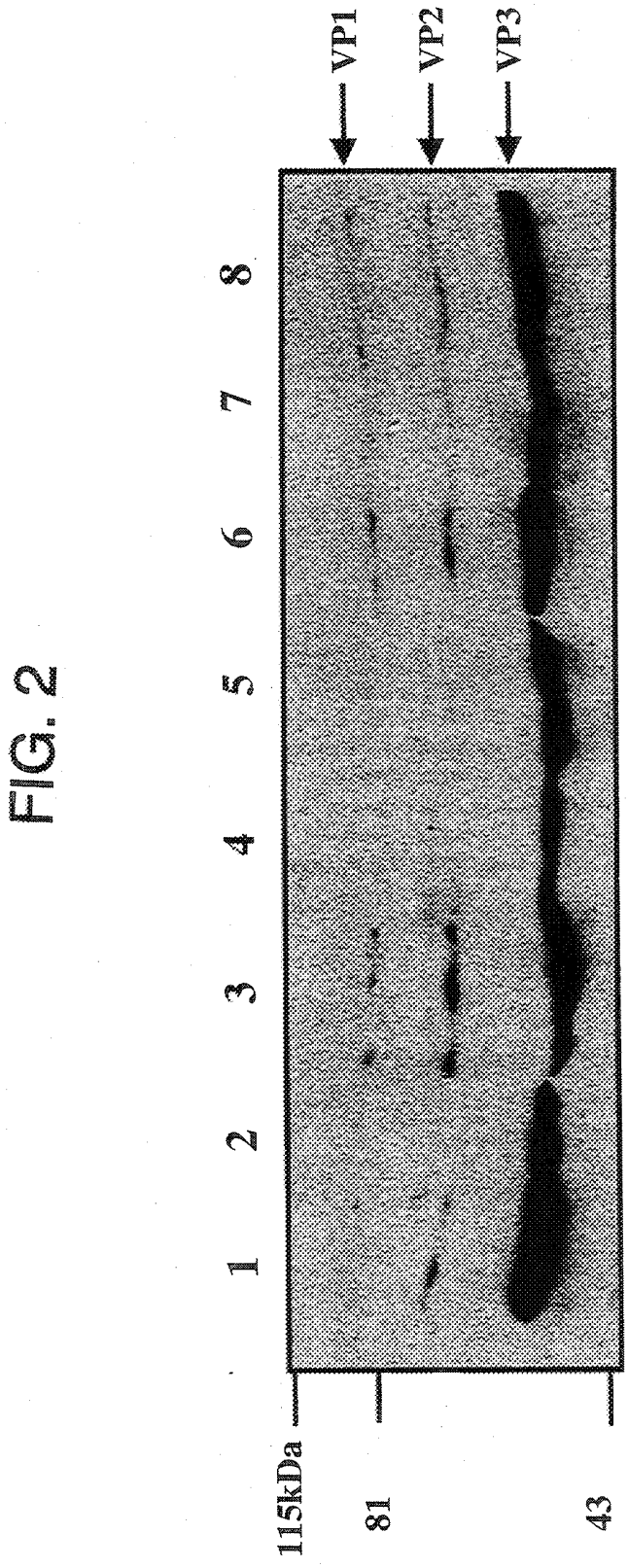


FIG. 3

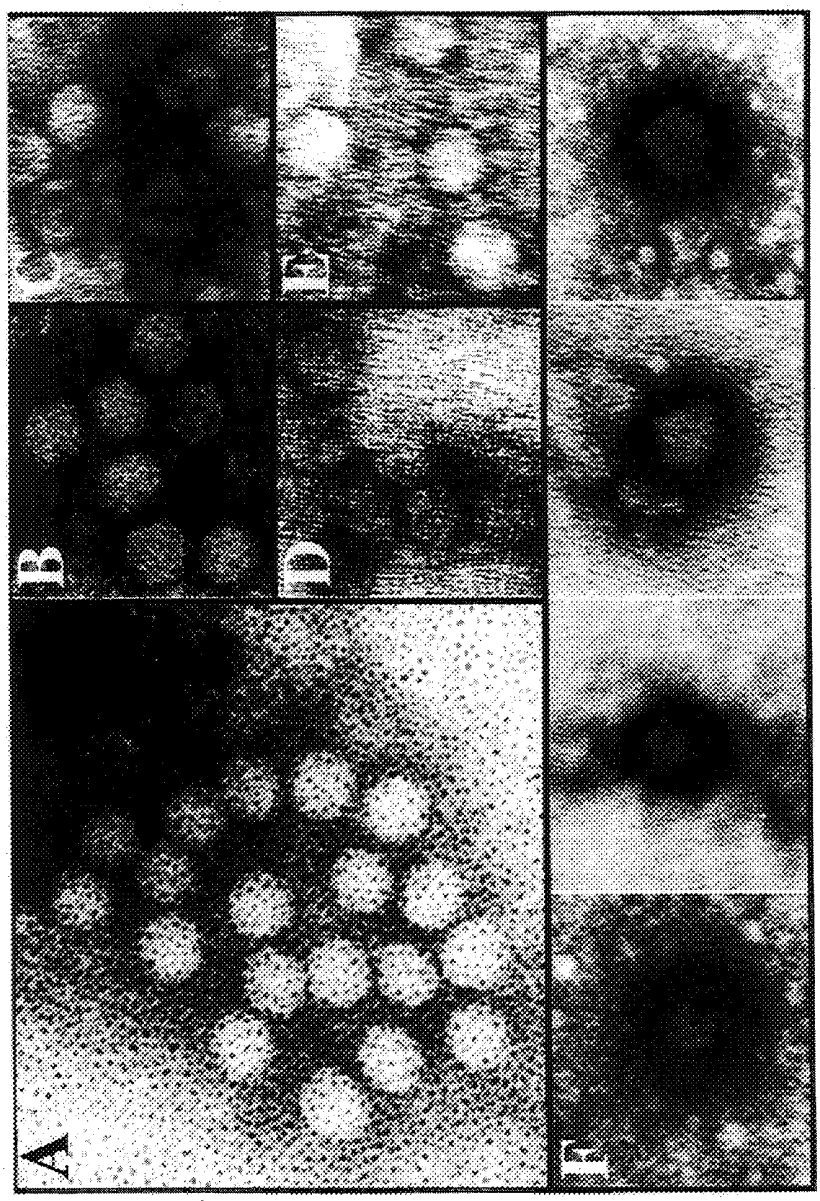


FIG. 4

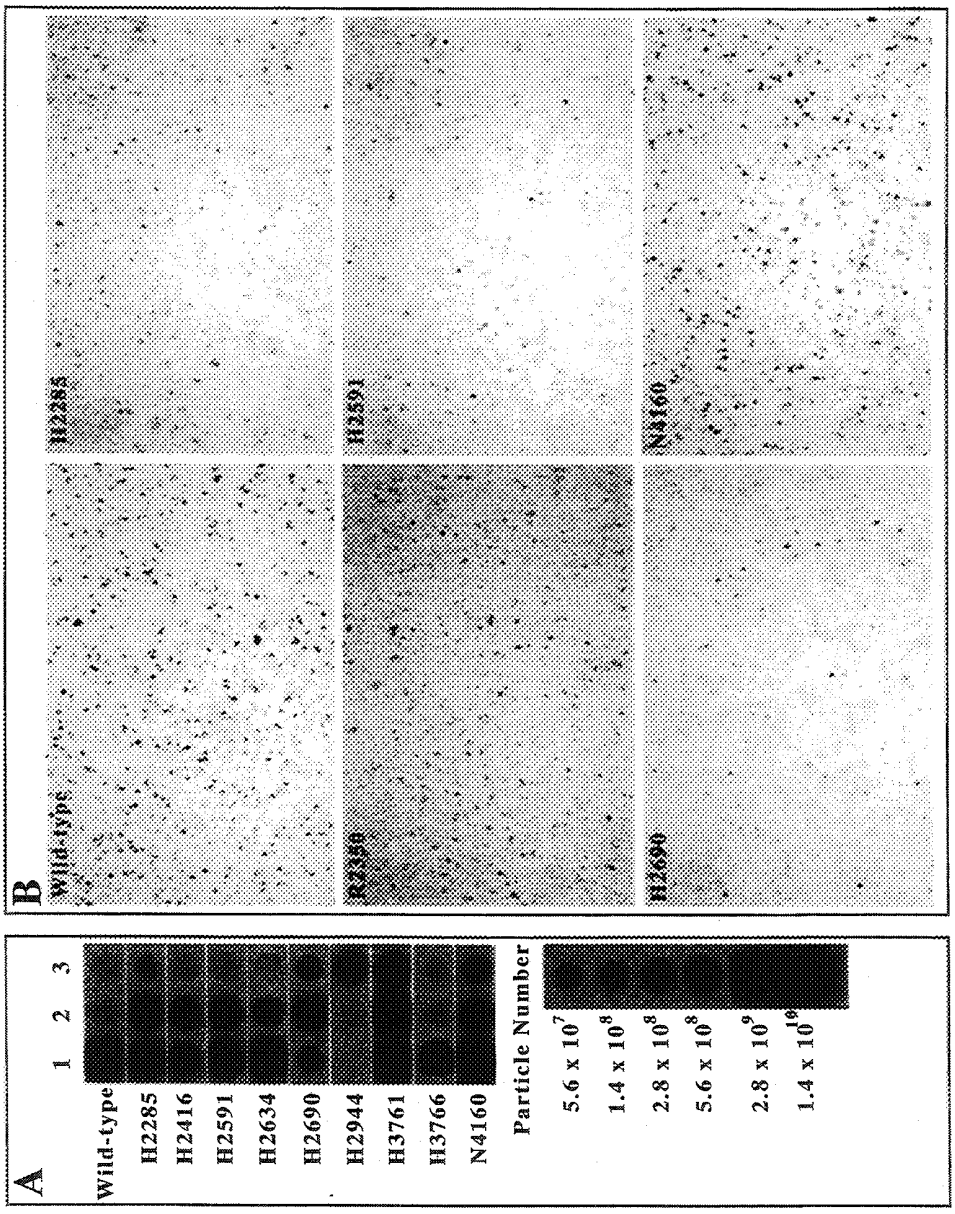
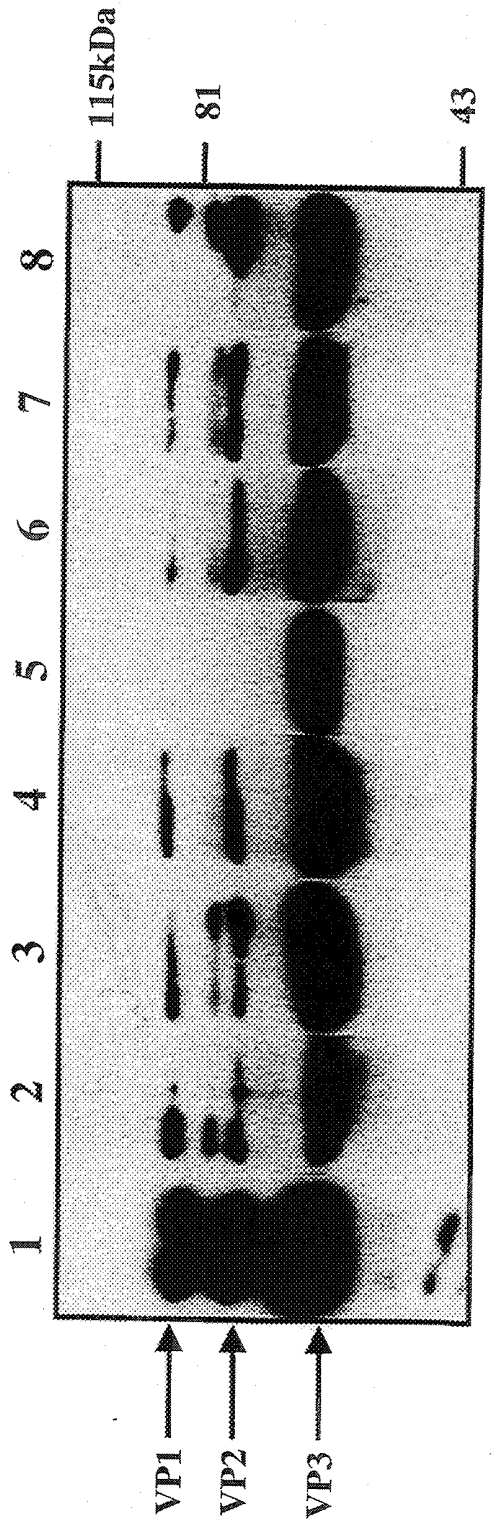

A
1 2 3
Wild-type
H2285
H2416
H2591
H2634
H2690
H2944
H3761
H3766
N4160
Particle Number
5.6 x 10^7
1.4 x 10^8
2.8 x 10^8
5.6 x 10^8
2.8 x 10^9
1.4 x 10^9
B
Wild-type
H2285
R2356
H2591
H2690
N4160

FIG. 5



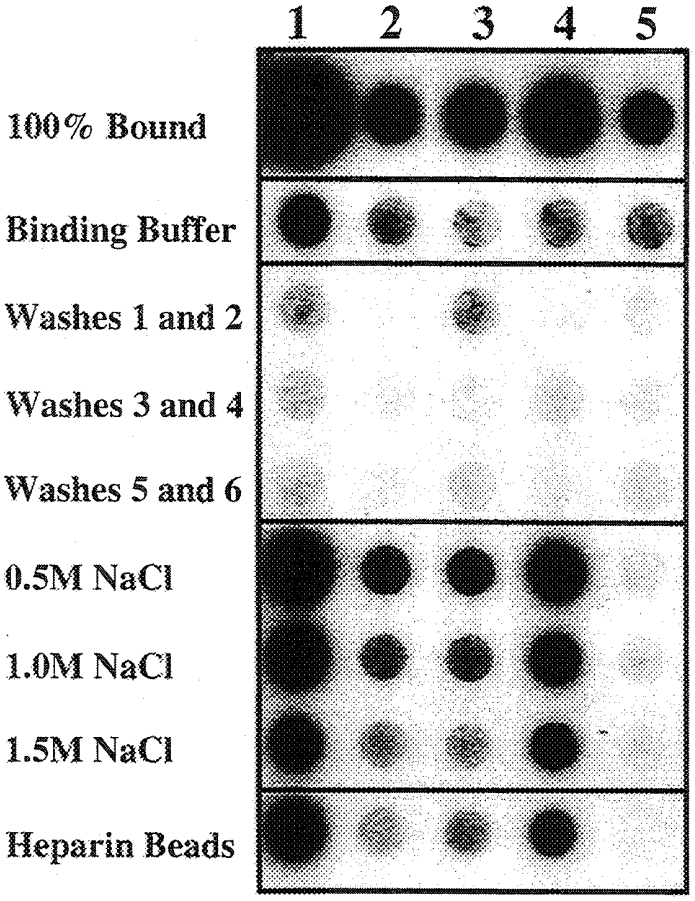

1
2
3
4
5
100% Bound
Binding Buffer
Washes 1 and 2
Washes 3 and 4
Washes 5 and 6
0.5M NaCl
1.0M NaCl
1.5M NaCl
Heparin Beads

FIG. 6

FIG. 9

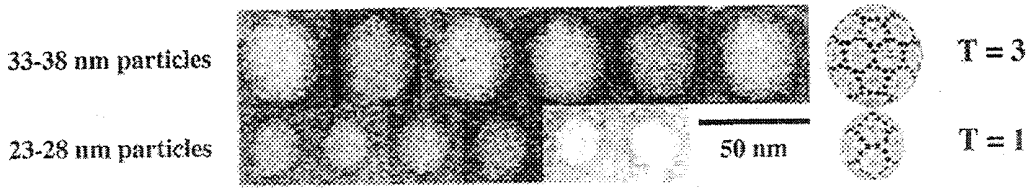

33-38 nm particles
23-28 nm particles
50 nm
T = 3
T = 1

FIG. 7

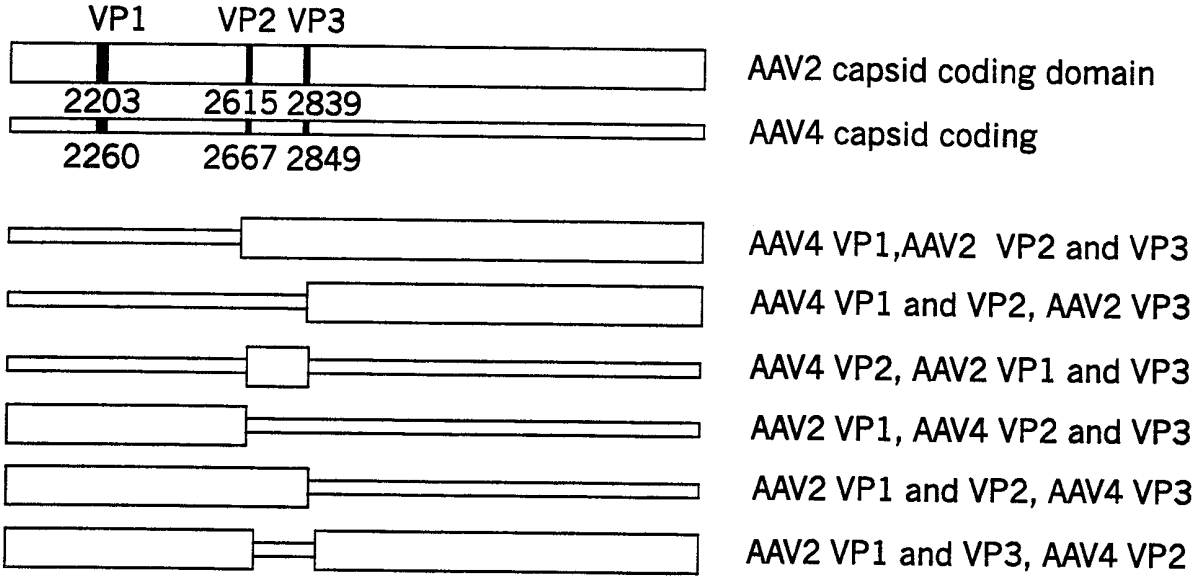
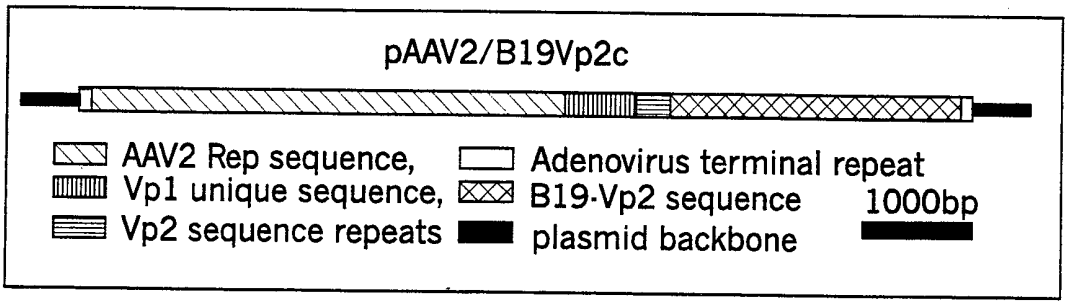

VP1
VP2 VP3
2203
2615 2839
2260
2667 2849
AAV2 capsid coding domain
AAV4 capsid coding
AAV4 VP1,AAV2 VP2 and VP3
AAV4 VP1 and VP2, AAV2 VP3
AAV4 VP2, AAV2 VP1 and VP3
AAV2 VP1, AAV4 VP2 and VP3
AAV2 VP1 and VP2, AAV4 VP3
AAV2 VP1 and VP3, AAV4 VP2

FIG. 8


pAAV2/B19Vp2c
AAV2 Rep sequence,
Vp1 unique sequence,
Vp2 sequence repeats
Adenovirus terminal repeat
B19-Vp2 sequence
plasmid backbone
1000bp

SEQUENCE LISTING

Appendix 1

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Appendix 2

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Appendix 3

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TACAACCACGCCGACGCGGAGTTTCAGGAGCGCCTTAAAGAAGATACGTCTTTTGG
GGGCAACCTCGGACGAGCAGTCTTCCAGGCGAAAAAGAGGGTTCTTGAACCTCTG
GGCCTGGTTGAGGAACCTGTTAAGACGGCTCCGGGAAAAAAGAGGCCGGTAGAGC
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CTGCAAGAAAAAGATTGAATTTTGGTCAGACTGGAGACGCAGACTCAGTACCTGAC
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Appendix 4

M A A D G Y L P D W L E D T L S E G I R Q W W K
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G Y K Y L G P F N G L D K G E P V N E A D A A A
L E H D K A Y D R Q L D S G D N P Y L K Y N H A
D A E F Q E R L K E D T S F G G N L G R A V
F Q A K K R V L E P L G L V E E P V K T A P G K K
R P V E H S P V E P D S S S G T G K A G Q Q P A
R K R L N F G Q T G D A D S V P D P Q P L G Q P
P A A P S G L G T N T M T S V N S A E A S T G A
G G G G S N S V K S M W S E G A T F S A N S V
T C T F S R Q F L I P Y D P E H H Y K V F S P A A
S S C H N A S G K E A K V C T I S P I M G Y S T
P W R Y L D F N A L N L F F S P L E F Q H L I E N
Y G S I A P D A L T V T I S E I A V K D V T D K T
G G G V Q V T D S T T G R L C M L V D H E Y K Y
P Y V L G Q G Q D T L A P E L P I W V Y F P P Q
Y A Y L T V G D V N T Q G I S G D S K K L A S E
E S A F Y V L E H S S F Q L L G T G G T A T M S
Y K F P P V P P E N L E G C S Q H F Y E M Y N P
L Y G S R L G V P D T L G G D P K F R S L T H E D
H A I Q P Q N F M P G P L V N S V S T K E G D S
S N T G A G K A L T G L S T G T S Q N T R I S L R
P G P V S Q P Y H H W D T D K Y V T G I N A I S
H G Q T T Y G N A E D K E Y Q Q G V G R F P N
E K E Q L K Q L Q G L N M H T Y F P N K G T Q Q
Y T D Q I E R P L M V G S V W N R R A L H Y E S
Q L W S K I P N L D D S F K T Q F A A L G G W G
L H Q P P P Q I F L K I L P Q S G P I G G I K S M
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R K A T G R W N P Q P G V Y P P H A A G H L P Y
V L Y D P T A T D A K Q H H R H G Y E K P E E L
W T A K S R V H P L *

Appendix 5

AATTCCCATCATCAATAATATACCTTATTTTGGATTGAAGCCAATATGATAATGAGGG
GGTGGAGTTTGTGACGTGGCGCGGGGCGTGGGAACGGGGCGGGTGACGTAGTAG
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GAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAA
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CCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGC
CTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTAC
CGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGA
GTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGC
GCGTTGGCCGATTCATTAATGCAG

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26505

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.

US CL :435/235.1, 320.1, 325.1, 456, 472; 424/93.2; 536/23.72, 23.4

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/235.1, 320.1, 325.1, 456, 472; 424/93.2; 536/23.72, 23.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	PONNAZHAGAN et al. Recombinant Human Parvovirus B19 Vectors: Erythroid Cell-Specific Delivery and Expression of Transduced Genes. Journal of Virology. June 1998. Vol. 72. No. 6. Pages 5224-5230, see entire document.	1-6, 10-17, 19, 20, 27-33, 36-41, 46-56, 60-67, 70, 112-115, 118, 119, 123, 124 --- 7-9, 22-26, 34, 35, 42, 44, 45, 57-59, 68, 69, 71-73, 79-90, 92-111, 116, 117, 120-122, 125-131, 133-168, 170-176, 178-191

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 24 FEBRUARY 2000	Date of mailing of the international search report 22 MAR 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MARY E. MOSHER, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26505

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RUTLEDGE et al. Infectious Clones and Vectors Derived from Adeno-Associated Virus (AAV) Serotypes Other Than AAV Type 2. Journal of Virology. January 1998, Vol. 72, No. 1, pages 309-319, see entire document.	7-9, 22-26, 45, 57-59, 68, 69, 71, 79-90, 92-111, 116, 117, 121, 122, 125-131, 133-146, 148, 150-162, 164, 166-168, 170-176, 178-182, 184-214, 216-229
Y	CHIORINI et al. Cloning of Adeno-Associated Virus Type 4 (AAV4) and Generation of Recombinant AAV4 Particles. Journal of Virology. September 1997, Vol. 71, No. 9, pages 6823-6833, see entire document.	7-9, 22-26, 45, 57-59, 68, 69,71, 79-90, 92-111, 116, 117, 121, 122, 125-131, 133-146, 148, 150-162, 164, 166-168, 170-176, 178-182, 184-214
Y	WO 98/09524 A1 (CHIRON CORPORATION) 12 March 1998, see entire document.	68, 69, 142, 225
Y	GAO et al. High-Titer Adeno-Associated Viral Vectors from a Rep/Cap Cell Line and Hybrid Shuttle Virus. Human Gene Therapy. 01 November 1998, Vol. 9, pages 2353-2362, see entire document.	34, 35, 44, 120, 191, 192, 203
Y	US 5,589,377 A (LEBKOWSKI et al) 31 December 1996, see claims 1-8.	42
X	HORIUCHI et al. Mapping determinants of the host range for canine cells in the genome of canine parvovirus using canine parvovirus/ mink enteritis virus chimeric viruses. Journal of General Virology. 1994, Vol. 75, pages 1319-1328, see entire document.	79, 80, 82, 102, 104, 107, 108, 146, 147, 149, 153-157
Y	WO 98/32842 A (GENETIC THERAPY, INC.) 30 July 1998, see entire document.	72, 73, 79-90, 92-111, 116, 117, 125-131, 133-168, 170-176, 178-191

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26505

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	YANG et al. Development of Novel Cell Surface CD34-Targeted Recombinant Adenoassociated Virus Vectors for Gene Therapy. Human Gene Therapy. 01 September 1998, Vol. 9, pages 1929-1937, see entire document.	146, 148, 151, 152, 154-164, 167-171, 173, 174, 181, 182, 184, 185, 187-190, 193-199, 201, 202, 204-214, 216-224, 226-229 ------- 147, 149, 150, 153, 165, 166, 172, 175-180, 183, 186, 191, 192, 200, 203, 225
Y	WO 96/00587 A1 (UNIVERSITY OF PITTSBURGH) 11 January 1996, see entire document.	232-235
Y	BROWN et al. Chimeric Parvovirus B19 Capsids for the Presentation of Foreign Epitopes. Virology. 1994, Vol. 198, pages 477-488, see entire document.	178, 179, 232-235
Y	CHAPMAN et al. Structure, Sequence, and Function Correlations among Parvoviruses. Virology. 1993, Vol. 194, pages 491-508, see Figure 3.	72, 73, 178, 179, 232-235
A	RABINOWITZ et al. Adeno-associated virus expression systems for gene transfer. Current Opinion in Biotechnology. October 1998, Vol. 9, No. 5, pages 470-475	1-235

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26505

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26505

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

C12N 7/01, 7/02, 15/86, 5/10, 15/00, 15/35, 15/62; A61K 35/76, 48/00

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

East USPATFULL, Medline, Derwent Biotechnology abs. Search terms: AAV, ADENOASSOCIAT?, ADENO(W)ASSOCIAT?. CHIMER?, CHIMAER?, HYBRID, HYBRIDS, CAP, CAPSID, COAT, FUSION, FUSED, FUSING, FUSE, PARVOVIR?, PARVO?, PARVO(W)(VIRUS OR VIRUSES OR VIRAL), PSEUDOTYP?, PSEUDO(W)TYP?, PSEUDO?, TARGET?, VP1, VP2, VP3, B19, VIRUS, SEROPOSITIVE, SEROTYPE, SEROTYPES, THERAPY, OVERCOM?, VECTOR? TYPE, TYPES, SERO(W)SWITCH, TAG, TAGGED, PURIF?, VIABLE, VIABILITY, INFECTIV?, POLYHIS?, HISTIDIN?, STABLE, STABLY, TS, TEMPERATURE(W)SENSITIV?, MIX?, RATIO, RATIOS, AFFINITY, ADENOVIRUS, REP, RECOMBINANT, BRADYKININ, LIGAND, TARGET?

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In addition, each group contains claims directed to more than one species of the generic invention. In order for all inventions and all of the species to be searched, the appropriate additional search fees must be paid.

Group 1, claim(s)1-71, 112-124, drawn to first product, pseudotyped parvovirus having AAV genome and heterologous parvovirus capsid, and associated intermediate products and methods of use.
The species are as follows:
Species A: B19 coat or other autonomous parvovirus,
Species B: AAV coat, different serotype from genome
The claims are deemed to correspond to the species listed above in the following manner:
Species A: 4, 5, 19, 20, 51,
Species B: 7-9, 22-26, 45, 57-59, 71, 122
The following group 1 claims are generic: 1-3, 6, 10-18, 20, 21, 27-50, 52-56, 60-70, 112-121, 123, 124

Group 2, claim(s) 72-111, 125-145, drawn to second product, parvovirus with chimeric capsid formed from plural parvovirus species, and associated intermediate products and methods of use.
The species are as follows:
Species C: Mixture of subunits from different parvoviruses
Species D: Capsid fusion protein combining regions from different parvoviruses
The claims are deemed to correspond to the species listed above in the following manner:
Species C: claims 76-78, 91, 132
Species D: claims 86-90
The following group 2 claims are generic: 72-75, 79-85, 92-102, 105-111, 125-131, 133-145

Group 3, claim(s) 146-235, drawn to third product, parvovirus with exogenous sequence in capsid protein, and associated intermediate products and methods of use.
The species are as follows:
Species E: exogenous targeting sequence
Species F: exogenous purification sequence
Species G: immunogenic sequence
The claims are deemed to correspond to the species listed above in the following manner:
Species E: claims 146-229
Species F: claims 230-231
Species G: claim 233
The following group 3 claims are generic: 232, 234, 235

The inventions listed as Groups 1-3, and the species listed above, do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The term "special technical features" is defined as "those technical features that define a contribution

which each of the claimed inventions, considered as a whole, makes over the prior art". In addition, if an international stage application claims multiple products, the first invention mentioned in the claims will be considered as the main invention in the claims. See 37 CFR 1.475, PCT article 17(3)(a) and section 1.476(c), MPEP 1805. In this application, Claim 1 defines the main invention. The main invention, as defined in claim 1, lacks a special technical feature, because claim 1 does not define over the prior art, being clearly anticipated by Ponnazhagan et al (Journal of Virology 5224-2530, issued June 1998. Since there is no feature that defines the main invention over the art, unity of invention is lacking.