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(54) **Title:** COMPOSITIONS AND METHODS FOR GENERATING ADENO-ASSOCIATED VIRAL VECTORS WITH UN-DETECTABLE CAPSID GENE CONTAMINATION

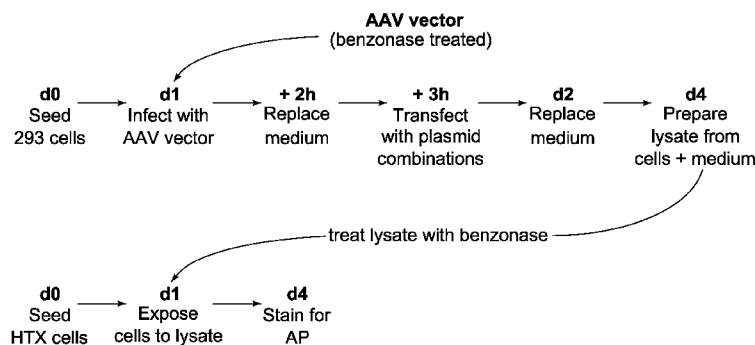


Fig.1.

(57) **Abstract:** In one aspect, the present invention provides an intron-modified cap expression cassette useful for generating ade-no-associated virus (AAV) vector particles. In another aspect, the present invention provides a method of reducing the immune re-sponse in a mammalian subject undergoing treatment with an AAV vector.

COMPOSITIONS AND METHODS FOR GENERATING ADENO-ASSOCIATED
VIRAL VECTORS WITH UNDETECTABLE CAPSID GENE CONTAMINATION

CROSS-REFERENCE(S) TO RELATED APPLICATION(S)

5 This application claims the benefit of U.S. Provisional Application
No. 61/412,237 filed on November 10, 2010, the disclosure of which is hereby
incorporated by reference in its entirety.

STATEMENT OF GOVERNMENT LICENSE RIGHTS

10 This invention was made with U.S. Government support under Grant
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STATEMENT REGARDING SEQUENCE LISTING

15 The sequence listing associated with this application is provided in text format in
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FIELD OF THE INVENTION

20 This invention relates to compositions and methods for generating
adeno-associated viral (AAV) vectors.

BACKGROUND

25 The prototype wild type AAV genome (AAV type 2) is a 4,600 base single
stranded DNA molecule including a terminal 145 base repeat sequence (ITR) at each end
(Muzyczka, *Curr. Top. Microbiol. Immunol.* 158:97–129, 1992). The AAV
non-structural and structural open reading frames (ORFs) responsible for replication and
encapsidation of the genome are derived from alternatively spliced transcripts originating
from three distinct promoters. The replication (Rep) proteins are designated as Rep 78/68
and Rep 52/40, initiated at the P5 and P19 promoters, respectively. The structural capsid
(Cap) proteins (VP1, VP2, and VP3) are under the control of the P40 promoter
30 (Muzyczka *supra*). A combination of the terminal repeats, cis-sequences proximal to the
AAV promoters, Rep proteins, adenovirus gene products, and cellular factors are
responsible for the appropriate expression of the AAV Rep and Cap proteins (Tratschin

et al., *Mol. Cell. Biol.* 6:2884-2894 (1986); Beaton et al., *J. Virol.* 63:4450-4454 (1989); Chang et al., *J. Virol.* 63:3479-3488 (1989); McCarty et al., *J. Virol.* 65:2936-2945 (1991); Shi et al., *Cell* 67:377-388 (1991); Horer et al., *J. Virol.* 69:5484-5496 (1995); Kyostio et al., *J. Virol.* 69:6787-6796 (1995); Lewis et al., *J. Virol.* 69:1628-1636 (1995);
5 Weger et al., *J. Virol.* 71:8437-8447 (1997); Ni et al., *J. Virol.* 72:2777-2787 (1998)).

Adeno-associated virus is a defective parvovirus that grows in cells in which certain functions are provided by a co-infecting helper virus. General reviews of AAV may be found in, for example, Carter, *Handbook of Parvoviruses*, Vol. I, pp. 169-228 (1989), and Berns, *Virology*, pp. 1743-1764, Raven Press, New York, N.Y. (1990),
10 incorporated herein by reference. Examples of co-infecting viruses that provide helper functions for AAV growth and replication are adenoviruses, herpes viruses, and in some cases, poxviruses such as vaccinia. The nature of the helper function is not entirely understood, but it appears that the helper virus indirectly renders the cell permissive for AAV replication. This belief is supported by the observation that AAV replication may
15 occur at low efficiency in the absence of helper virus co-infection if the cells are treated with agents that are either genotoxic or that disrupt the cell cycle.

Although AAV may replicate to a limited extent in the absence of helper virus in these unusual conditions, more generally infection of cells with AAV in the absence of helper functions results in the proviral AAV genome integrating into the host cell
20 genome. If these cells are superinfected with a helper virus such as adenovirus, the integrated AAV genome can be rescued and replicated to yield a burst of infectious progeny AAV particles. The fact that integration of AAV appears to be efficient suggests that AAV would be a useful vector for introducing genes into cells for use, such as, in human gene therapy.

25 AAV has a very broad host range without any obvious species or tissue specificity and can replicate in virtually any cell line of human, simian or rodent origin provided that an appropriate helper is present. AAV is also relatively ubiquitous and has been isolated from a variety of animal species including most mammalian and several avian species.

AAV vectors including heterologous polynucleotide sequences flanked by the
30 AAV terminal repeats (ITRs) can be assembled into virions when introduced into appropriate cells by transfection with packaging plasmids encoding the Rep and Cap reading frames, but lacking the ITRs, and by co-infection with the non-related helper

adenovirus (Samulski et al., *J. Virol.* 63:3822-3828 (1989); Muzcyka, *Curr. Top. Microbiol. Immunol.* 158:97-129 (1992)).

Initial AAV packaging plasmids contained simple deletions of the packaging signal (ITRs) to supply the Rep and Cap proteins for AAV vector production without generating wild type virus (Samulski et al., *supra* (1989); Muzcyzka, *supra* (1992)). The AAV/Ad packaging construct, for example, has been widely used to generate AAV vector stocks by co-transfection into human embryonic kidney 293 cells (Graham et al., *J. Virol.* 36:59-74 (1977); Samulski et al., *J. Virol.* 63:3822-3828 (1989)). A derivative packaging plasmid, ACG-2, was described which converted the Rep 79/68 initiation codon from AUG to ACG to reduce the level of Rep 78/68 expression (Li et al., *J. Virol.* 71:5236-5243 (1987)). Over expression of the Rep 78/68 proteins has been correlated with decreases in AAV vector production. Expression of the AAV reading frames from these plasmids is still dependent upon transcription from the AAV P5, P19, and P40 promoters.

Co-transfection of AAV/Ad and AAV vector plasmids has been shown to result in significant contamination of vector stocks with replication-competent AAV (rcAAV) presumably by recombination between the plasmids during transfection (Allen et al., *J. Virol.* 71:6816-6822 (1997); Salvetti et al., *Hum. Gene Ther.* 9:695-706 (1998); Wang et al., *J. Virol.* 72:5472-5480 (1998)). An AAV packaging plasmid has previously been described which split the AAV rep and cap genes, placing them in an inverted orientation relative to the wild type AAV, and replaced the P5 and P40 promoters with the mouse metallothionein and cytomegalovirus (CMV) regulatory sequences, respectively (MTrep/CMVcap, see FIG. 1 of Allen et al., *J. Virol.* 71:6816-6822 (1997); WO98/27204, each hereby incorporated herein by reference). AAV vector production with this packaging plasmid was comparable to levels of vector produced with the AAV/Ad packaging plasmid and was free of rcAAV. Production of AAV vector using MTrep/CMVcap was still enhanced by infection with helper adenovirus despite the substitution of the P5 and P40 promoter sequences.

Adenovirus infection is an easy and efficient method for supplying the helper activities necessary for AAV vector production. However, removal of adenovirus from AAV vector stocks is laborious and can result in the contamination of vector stocks with potentially immunoreactive adenovirus proteins. Recently it has been shown that the adenovirus activities required for AAV vector production can be supplied by transfection

of 293 cells with plasmids containing the E2A, E4 and VA RNA genes (Xiao et al., Journal of Virology, 72:2224-2232 (1998); Matsushita et al., *Gene Ther.* 5:938-945 (1998)). Full helper activity required the transfection of all three transcription units (Matsushita et al., *supra* (1998), incorporated herein by reference).

5 There are at least two desirable features of any AAV vector designed for use in human gene therapy. First, the transducing vector must be generated at titers sufficiently high to be practicable as a delivery system. This is especially important for gene therapy strategies aimed at *in vivo* delivery of the vector. For example, it is likely that for many desirable applications of AAV vectors, such as treatment of cystic fibrosis by direct *in vivo* delivery to the airway, the required dose of transducing vector may be in excess of 10¹⁰ particles. Secondly, the vector preparations must be essentially free of wild type AAV virus (or any replication-competent AAV). The attainment of high titers of AAV vectors has been difficult for several reasons including preferential encapsidation of wild type AAV genomes (if they are present or generated by recombination), and the difficulty in generating sufficient complementing functions such as those provided by the wild type rep and cap genes. Useful cell lines expressing such complementing functions have been especially difficult to generate, in part because of pleiotropic inhibitory functions associated with the rep gene products. Thus, cells in which the rep gene is integrated and expressed tend to grow slowly or express Rep at very low levels.

20 Vectors based on adeno-associated viruses (AAV) have been used in many applications *in vivo* because they promote persistent gene expression in dividing and non-dividing cells in multiple somatic tissues of animals (see, e.g., Herzog R.W. et al., *Nat Med* 5:56-63 (1999); Passini MA et al., *J Virol* 77:7034-7040 (2003); Weber M et al., *Mol Ther* 7:774-781 (2003) and Mochizuki S et al., *Gene Ther* 11:1081-1086 (2004)). However, recent studies have shown that the lack of an immune response seen in many mouse and some large animal studies has not been duplicated in human trials. For example, in a clinical trial for hemophilia B, two of seven subjects given an AAV vector-expressing clotting factor IX (FIX) developed a transient self-limiting increase in liver transaminases, followed by clearance of the FIX-expressing cells at 4 to 8 weeks after delivery (Manno, C.S., et al., *Nat. Med.* 12:342-347 (2006)). In this clinical trial for hemophilia B, a cytotoxic lymphocyte (CTL) response to AAV capsid but not to FIX was detected in peripheral blood mononuclear cells. In another clinical trial involving lipoprotein lipase, clearance of AAV-transduced cells coincided with a CTL

response towards the capsid, but not the transgene (Mingozzi, F., et al., *Blood* 114:2077-2086 (2009)).

Therefore, a need exists for generating AAV vector preparations that reduce or eliminate the immune response in a mammalian subject.

5

SUMMARY

In one aspect, the present invention provides an intron-modified cap expression cassette useful for generating adeno-associated virus (AAV) vector particles. The intron-modified cap expression cassette comprises: (i) a nucleic acid molecule comprising a nucleotide sequence encoding AAV capsid protein operably linked to a promoter, wherein the encoded AAV capsid protein is capable of packaging AAV vectors in the presence of rep and adeno helper functions, and (ii) at least one heterologous intron sequence operably linked to the nucleotide sequence encoding the AAV capsid protein; wherein the total length of the expression cassette is at least 5 kilobases. In one embodiment, the invention provides a vector comprising the intron-modified cap expression cassette. In another embodiment, the invention provides a cell line comprising the intron-modified cap expression cassette.

In another aspect, the present invention provides a method of generating an expression cassette for producing adeno-associated virus (AAV) vectors, comprising inserting at least one heterologous intron sequence into a nucleic acid molecule comprising a nucleotide sequence encoding an AAV capsid protein operably linked to a promoter, wherein the total length of the expression cassette is at least 5 kilobases.

In another aspect, the present invention provides a method of reducing intact cap gene contamination in a population of AAV vector particles. The method comprises: (a) introducing an AAV vector into a suitable host cell comprising an intron-modified cap expression cassette; (b) expressing rep and adeno helper functions in the host cell; and (c) culturing the host cell to produce a population of AAV vector particles.

In another aspect, the present invention provides a method of reducing the immune response in a mammalian subject undergoing treatment with an AAV vector comprising administering to the mammalian subject a preparation of AAV vector particles comprising a nucleic acid sequence encoding a therapeutic molecule, wherein the preparation of AAV vector particles was produced with an intron-modified cap expression cassette, and wherein the immune response in the mammalian subject to the AAV vector generated using the intron-modified cap expression cassette is significantly

reduced or absent as compared to the immune response generated with a preparation of AAV vector particles generated using a standard cap expression cassette.

In another aspect, the present invention provides a complementation assay for measuring the presence or amount of cap gene contamination in a preparation of AAV vector virions, the assay comprising: (a) infecting a host cell with a test preparation of AAV vector, wherein the host cell comprises: (i) an indicator AAV vector with a detectable marker, (ii) a rep expression vector, and (iii) adenovirus helper functions; (b) culturing the host cell for a period of time sufficient to generate AAV vector virions from the indicator AAV vector; (c) contacting an indicator cell with cell lysate obtained from the host cells cultured in accordance with step (b); and (d) determining the presence or amount of indicator cells that are positive for the detectable marker, wherein a positive result indicates that the test preparation of AAV vector virions contains cap gene contamination.

In another aspect, the present invention provides a complementation assay for measuring the presence or amount of rep gene contamination in a preparation of AAV vector virions, the assay comprising: (a) infecting a host cell with a test preparation of AAV vector, wherein the host cell comprises: (i) an indicator AAV vector with a detectable marker, (ii) a cap expression vector, and (iii) adenovirus helper functions; (b) culturing the host cell for a period of time sufficient to generate AAV vector virions from the indicator AAV vector; (c) contacting an indicator cell with cell lysate obtained from the host cells cultured in accordance with step (b); and (d) determining the presence or amount of indicator cells that are positive for the detectable marker, wherein a positive result indicates that the test preparation of AAV vector virions contains rep gene contamination.

As described herein, the compositions of the invention are useful for carrying out the methods of the invention.

DESCRIPTION OF THE DRAWINGS

The foregoing aspects and many of the attendant advantages of this invention will become more readily appreciated as the same become better understood by reference to the following detailed description, when taken in conjunction with the accompanying drawings, wherein:

FIGURE 1 is a flow diagram illustrating the steps of a cap and rep complementation assay, as described in Example 2;

FIGURE 2A illustrates representative expression cassettes comprising a nucleotide sequence encoding the cap6 ORF with and without a heterologous intron inserted upstream of the cap open reading frame, as described in Example 3;

FIGURE 2B is a Western blot of cell lysates of 293T cells transfected with 10 µg DNA using an anti-AAV VP1+VP2+VP3 mouse monoclonal antibody, with 10 µl lysate loaded per lane; showing cap expression resulting from the cap6 expression cassettes illustrated in FIGURE 2A, as described in Example 3; and

FIGURE 2C illustrates the elements of a representative expression cassette comprising a nucleotide sequence encoding the cap6 ORF, designated "pCMVE2cap6," as described in Example 3.

DESCRIPTION OF SEQUENCE LISTING

SEQ ID NO:1 is the AAV-6 CAP ORF.
 SEQ ID NO:2 is the AAV-6 Capsid protein encoded by SEQ ID NO:1;
 SEQ ID NO:3 is a portion of the AAV-6 intron only including SA1 and SA2.
 SEQ ID NO:4 is the AAV-6 intron including SA1 and SA2.
 SEQ ID NO:5 is the EIF2S1 intron region 4285nt.
 SEQ ID NO:6 is the AAV-6 intron starting at SA1 through the CAP ORF.
 SEQ ID NO:7 is the complete sequence of CMVE2cap6.
 SEQ ID NO:8 is the AAV-2 cap ORF (CDS 2203-4410 from NC_001410.2).
 SEQ ID NO:9 is the AAV-2 Capsid protein encoded by SEQ ID NO:8.
 SEQ ID NO:10 is Rep forward primer.
 SEQ ID NO:11 is Rep reverse R1 primer.
 SEQ ID NO:12 is CapF forward primer.
 SEQ ID NO:13 is CapR540 primer.
 SEQ ID NO:14 is primer cFIXF1.
 SEQ ID NO:15 is primer cFIXR2.
 SEQ ID NO:16 is primer lacZqF1.
 SEQ ID NO:17 is primer lacZqR1.
 SEQ ID NO:18 is primer EIF2S1inFN.
 SEQ ID NO:19 is primer EIF2S1inRN.

DETAILED DESCRIPTION

Unless specifically defined herein, all terms used herein have the same meaning as they would to one skilled in the art of the present invention. The practice of the

subject matter described herein will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, cell biology and recombinant DNA, which are within the skill of the art. See, e.g., Sambrook, Fritsch, and Maniatis, *Molecular Cloning: a Laboratory Manual*, 2nd edition (1989); *Current Protocols in Molecular Biology* (F.M. Ausubel et al. eds., 1987); the series *Methods in Enzymology* (Academic Press, Inc.); *PCR 2: A Practical Approach* (M. Jr McPherson, B.D. Hames, and G.R. Taylor, eds., 1995); and *Animal Cell Culture* (R.I. Freshney, ed., 1987).

The following definitions are provided in order to provide clarity with respect to the terms as they are used in the specification and claims to describe the present invention.

As used herein, the term "vector" is a nucleic acid molecule which transfers and/or replicates an inserted nucleic acid molecule into and/or between host cells. In some embodiments, the vectors of the invention are incapable of autonomous self-replication.

As used herein, the term "nucleic acid sequences allowing for autonomous replication" refers to a polynucleotide comprising an origin of replication (generally referred to as an ori sequence) which allows for replication of the polynucleotide in the appropriate host cell.

As used herein, the term "heterologous" means derived from a genotypically distinct entity from that of the rest of the entity to which it is compared. For example, a polynucleotide introduced by genetic engineering techniques into a different cell type is a heterologous polynucleotide. When that polynucleotide is expressed, the polynucleotide can encode a heterologous polypeptide.

As used herein, the term "operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. For example, a promoter sequence is operably linked to a coding sequence if the promoter sequence promotes transcription of the coding sequence. As another example, an intron sequence is operably linked to a transcriptional unit if the intron contains splice donor and splice acceptor sites allowing for proper splicing of the transcription unit. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame. However, since enhancers may function when separated from the

promoter by several kilobases and intronic sequences may be of variable length, some nucleotide sequences may be operably linked but not contiguous.

As used herein, the term "expression cassette" refers to a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements which permit transcription of a particular nucleic acid in a host cell. The expression cassette can be incorporated into a plasmid, chromosome, virus, or nucleic acid fragment.

As used herein, the term "percent identity" or "percent identical", refers to the percentage of amino acid residues in a polypeptide sequence, or nucleic acids in a nucleotide sequence that are identical with the amino acid sequence or nucleic acid sequence, respectively, of a specified molecule, after aligning the sequences to achieve the maximum percent identity. For example, the Vector NTI Advance™ 9.0 may be used for sequence alignment. Techniques for determining nucleic acid and amino acid "sequence identity" also are known in the art. Typically, such techniques include determining the nucleotide sequence of the mRNA for a gene and/or determining the amino acid sequence encoded thereby, and comparing these sequences to a second nucleotide or amino acid sequence. In general, "identity" refers to an exact nucleotide-to-nucleotide or amino acid-to-amino acid correspondence of two polynucleotides or polypeptide sequences, respectively. Two or more sequences (polynucleotide or amino acid) can be compared by determining their "percent identity." The percent identity of two sequences, whether nucleic acid or amino acid sequences, is the number of exact matches between two aligned sequences divided by the length of the shorter sequences and multiplied by 100. Percent identity may also be determined, for example, by comparing sequence information using the advanced BLAST computer program, including version 2.2.9, available from the National Institutes of Health. The BLAST program is based on the alignment method of Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 87:2264-2268 (1990) and as discussed in Altschul et al., *J. Mol. Biol.* 215:403-410 (1990); Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 90:5873-5877 (1993); and Altschul et al., *Nucleic Acids Res.* 25:3389-3402 (1997). Ranges of desired degrees of sequence identity are approximately 80% to 100% and integer values there between. Typically, the percent identities between a disclosed sequence and a claimed sequence are at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%.

Alternatively, the degree of sequence similarity between polynucleotides can be determined by hybridization of polynucleotides under conditions that form stable

duplexes between homologous regions, followed by digestion with single-stranded-specific nuclease(s), and size determination of the digested fragments. Two DNA, or two polypeptide sequences are "substantially homologous" to each other when the sequences exhibit at least about 80-85%, preferably 85-90%, more preferably 5 90-95%, and most preferably 98-100% sequence identity to the reference sequence over a defined length of the molecules, as determined using the methods above. As used herein, substantially homologous also refers to sequences showing complete identity to the specified DNA or polypeptide sequence. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, 10 stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al., *supra*; DNA Cloning, *supra*; Nucleic Acid Hybridization, *supra*.

Additionally, as defined herein, a nucleotide sequence is intended to refer to a natural or synthetic linear and sequential array of nucleotides and/or nucleosides, and 15 derivatives thereof. The terms "encoding" and "coding" refer to the process by which a nucleotide sequence, through the mechanisms of transcription and translation, provides the information to a cell from which a series of amino acids can be assembled into a specific amino acid sequence to produce a polypeptide.

As used herein, the term "AAV" is a standard abbreviation for adeno-associated 20 virus. Adeno-associated virus is a single-stranded DNA parvovirus that grows only in cells in which certain functions are provided by a co-infecting helper virus. There are currently thirteen serotypes of AAV that have been characterized, as shown below in Table 1. General information and reviews of AAV can be found in, for example, Carter, 1989, *Handbook of Parvoviruses*, Vol. 1, pp. 169-228, and Berns, 1990, *Virology*, 25 pp. 1743-1764, Raven Press, (New York). The type 6 and type 2 AAV serotypes have been used to illustrate the present invention in the Examples provided herein below. However, it is fully expected that these same principles will be applicable to additional AAV serotypes since it is well known that the various serotypes are quite closely related, both structurally and functionally, even at the genetic level. (See, for example, 30 Blacklowe, 1988, pp. 165-174 of *Parvoviruses and Human Disease*, J.R. Pattison, ed.; and Rose, *Comprehensive Virology* 3:1-61 (1974)). For example, all AAV serotypes apparently exhibit very similar replication properties mediated by homologous rep genes; and all bear three related capsid proteins such as those expressed in AAV 6. The degree

of relatedness is further suggested by heteroduplex analysis which reveals extensive cross-hybridization between serotypes along the length of the genome; and the presence of analogous self-annealing segments at the termini that correspond to "inverted terminal repeat sequences" (ITRs). The similar infectivity patterns also suggest that the replication
5 functions in each serotype are under similar regulatory control.

An "AAV vector" as used herein refers to a vector comprising one or more polynucleotides of interest (or transgenes) that are flanked by AAV terminal repeat sequences (ITRs). Such AAV vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been transfected with a vector encoding
10 and expressing rep and cap gene products, i.e., AAV Rep and Cap proteins, and wherein the host cell has been transfected with a vector which encodes and expresses a protein from the adenovirus open reading frame E4orf6. When an AAV vector is incorporated into a larger polynucleotide, e.g., in a chromosome or in another vector such as a plasmid used for cloning or transfection, then the AAV vector is typically referred to as a
15 "pro-vector". The pro-vector can be "rescued" by replication and encapsidation in the presence of AAV packaging functions and necessary helper functions provided by E4orf6.

An "AAV virion" or "AAV viral particle" or "AAV vector particle" refers to a viral particle composed of at least one AAV capsid protein (preferably by all of the
20 capsid proteins of a particular AAV serotype) and an encapsidated polynucleotide AAV vector. If the particle comprises a heterologous polynucleotide (i.e. a polynucleotide other than a wild-type AAV genome such as a transgene to be delivered to a mammalian cell), it is typically referred to as an "AAV vector particle" or simply an "AAV vector". Thus, production of AAV vector particle necessarily includes production
25 of AAV vector, as such a vector is contained within an AAV vector particle.

"Packaging" as used herein refers to a series of subcellular events that results in the assembly and encapsidation of a viral vector, particularly an AAV vector. Thus, when a suitable vector is introduced into a packaging cell under appropriate conditions it can be assembled into a viral particle. Functions associated with packaging of viral
30 vectors, particularly AAV, are described herein and in the art.

AAV "rep" and "cap" genes are genes encoding replication and encapsidation proteins, respectively. AAV rep and cap genes have been found in all AAV serotypes examined to date, and are described herein and in the references cited. In wild-type

AAV, the rep and cap genes are generally found adjacent to each other in the viral genome (i.e., they are "coupled" together as adjoining or overlapping transcriptional units), and they are generally conserved among AAV serotypes. AAV rep and cap genes are also individually and collectively referred to as "AAV packaging genes." The AAV cap gene in accordance with the present invention encodes a Cap protein which is capable of packaging AAV vectors in the presence of rep and adeno helper function and is capable of binding target cellular receptors. In some embodiments, the AAV cap gene encodes a capsid protein having an amino acid sequence derived from a particular AAV serotype, for example the serotypes shown in Table 1. In some embodiments, the AAV cap gene encodes a hybrid capsid protein comprising an amino acid sequence comprising a first region that is derived from a first AAV serotype and at least a second region that is derived from a second AAV serotype. In some embodiments, the AAV cap gene encodes a capsid protein variant, having at least 70%, (such as at least 80%, at least 85%, at least 90%, at least 95% or at least 98% identity with the nucleotide sequence encoding the cap ORF in its respective AAV serotype.

As used herein, the terms "host cells," "cell lines," "cell culture," "packaging cells," and other such terms describe higher eukaryotic cells, preferably mammalian cells, most preferably human cells, useful in the present invention. These cells can be used as recipients for recombinant vectors, viruses or other transfer polynucleotides, and include the progeny of the original cell that was transduced. It is understood that the progeny of a single cell may not necessarily be completely identical, in, for example, morphology or in genomic complement, to the original parental cell.

As described herein, the inventors have determined that a correlation exists between the presence of cap gene contaminants in packaged AAV vector preparations and the immunogenicity of such AAV vectors when administered to a mammalian subject. As described in Example 1, the inventors discovered that AAV capsids can package DNA that does not contain AAV ITR sequences, and show that the packaged DNA can express protein in vector-transduced cells. As described in Example 2, consistent with the results observed in Example 1, the inventors were able to detect cap and rep DNA in several AAV vectors made using standard techniques (i.e., cap expression vectors not modified with a heterologous intron) and found expression of Cap proteins in cells transduced with standard AAV vector preparations by using a sensitive complementation assay.

Accordingly, the present invention provides compositions comprising an intron-modified cap expression cassette, wherein the expression cassette is at least 5 kilobases in length (cap + intron, also referred to as "captron") and methods of using the same for generating adeno-associated virus (AAV) vector particles with reduced or undetectable levels of cap gene contamination. As described in Example 3, the inventors generated a novel intron-modified cap expression cassette (captron construct) which eliminates capsid gene transfer and capsid expression in transduced cells. As described in Example 4, the inventors demonstrated that AAV vectors generated using the captron construct are dramatically less immunogenic when administered to a dog at therapeutic *in vivo* amounts than AAV vectors generated using the standard cap6 construct that is not modified with a heterologous intron. In view of the results demonstrated herein, it is expected that AAV vectors produced using captron plasmids will generate an increase in the number of transduced cells and *in vivo* persistence of a therapeutic transgene of interest. It is also expected that AAV-captron generated vectors will reduce the level of, or eliminate the need for, an immunosuppressive regimen after AAV vector transduction in a mammalian subject.

In accordance with the foregoing, in one aspect, the invention provides an intron-modified cap expression cassette (captron) useful for generating adeno-associated virus (AAV) vector particles. The intron-modified cap expression cassette in accordance with this aspect of the invention comprises: (i) a nucleic acid molecule comprising a nucleotide sequence encoding AAV capsid protein operably linked to a promoter, wherein the encoded AAV capsid protein is capable of packaging AAV vectors in the presence of rep and adeno helper functions, and (ii) at least one heterologous intron sequence operably linked to the nucleotide sequence encoding the AAV capsid protein; wherein the total length of the expression cassette is at least 5 kilobases.

FIGURES 2A (pCMVE2cap6) and 2C illustrate exemplary embodiments of an intron-modified cap expression cassette (10) in accordance with this aspect of the invention. As shown in FIGURE 2C, the expression cassette (10) includes a nucleotide sequence encoding an AAV capsid protein (cap open reading frame (ORF)) (40), operably linked to a promoter (20), and at least one heterologous intron sequence (30) operably linked to the nucleotide sequence encoding the AAV Cap protein (40).

In accordance with this aspect of the invention, the expression cassette 10 includes a nucleotide sequence encoding an AAV capsid protein that is capable of

packaging AAV vectors in the presence of rep and adeno helper functions. The expression cassette 10 may include a nucleotide sequence encoding a cap ORF from any AAV serotype, including AAV serotypes that are human or non-human. At least 13 AAV serotypes have been identified, cloned and sequenced, and at least 100 new AAV variants have been isolated from non-primates, primates and humans. Exemplary serotypes are described in TABLE 1 and include Serotypes AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAV-8, AAV-9, AAV-10, AAV-11, AAV-12 and AAV-13. In a particularly preferred embodiment, the present invention provides an expression cassette comprising a nucleotide sequence encoding AAV type 2 cap ORF (SEQ ID NO:8), or AAV type 6 cap ORF (SEQ ID NO:1).

In some embodiments, the expression cassette comprises a nucleotide sequence encoding a cap ORF variant sequence, the variant sequence having at least 70% sequence identity, more preferably at least about 80%, more preferably at least 85%, at least 90%, at least 95% or at least 98% sequence identity with the nucleotide sequence encoding the Cap ORF in its respective AAV serotype. In one embodiment, the expression cassette comprises a nucleotide sequence encoding a cap ORF variant sequence having at least 70% sequence identity, more preferably at least about 80%, more preferably at least 85%, at least 90%, at least 95% or at least 98% sequence identity with the nucleotide sequence encoding the Cap ORF from AAV serotype 2 (SEQ ID NO:8) or AAV serotype 6 (SEQ ID NO:1).

In some embodiments, the expression cassette comprises a nucleotide sequence encoding a cap ORF variant sequence with regions or domains or individual amino acids that are derived from two or more different serotypes (also referred to as a chimeric or hybrid AAV capsid), wherein the cap ORF variant sequence is capable of packaging AAV vectors in the presence of rep and adeno helper function and is capable of binding target cellular receptors. Exemplary chimeric AAV variant capsid sequences suitable for use in the present invention are described in U.S. Patent Publication No. 2010/0047174, hereby incorporated herein by reference. As described in U.S. Patent Publication No. 2010/0047174, an alignment of the amino acid sequences of various capsid proteins shows conservation of the five known loops on the exterior of the AAV capsid which are likely to be involved in capsid binding to cellular receptors and recognized by neutralizing antibodies. Accordingly, in a preferred embodiment the cap ORF variant

sequence for use in the present invention comprises the five known loop regions derived from one or more different serotypes, and is capable of binding to cellular receptors.

It has been demonstrated that AAV vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been transfected with a vector encoding and expressing rep and cap gene products, i.e., AAV Rep and Cap proteins, and wherein the host cell has been transfected with a vector which encodes and expresses a protein from the adenovirus open reading frame E4orf6. It has been further demonstrated that rep and cap transcription units may be provided as two separate plasmids relative to single AAV packaging plasmids. Presentation on separate plasmids apparently improves the function of the heterologous promoters to initiate transcription of the associated polynucleotide. See, e.g., U.S. Patent No. 7,208,315 and U.S. Patent No. 6,541,258, each hereby incorporated herein by reference. For example, as described in U.S. Patent No. 6,541,258, an AAV "split-cap" packaging system is described which utilizes a recombinant gene encoding one or more AAV Cap proteins, which gene is separated from rep-specific sequences of an AAV rep gene, i.e., rep78. The split-cap gene is operably linked to a promoter, preferably a heterologous promoter, i.e., a promoter other than the AAV P40 promoter, which heterologous promoter is incorporated upstream of the split-cap coding region, either in place of or in addition to the P40 promoter.

Although the split packaging system (e.g., using a plasmid called "CMV-cap" as described in U.S. Patent No. 6,541,258, and illustrated in FIGURE 2A), is capable of generating high titer AAV vector particles, as demonstrated in Example 2 herein, cap and rep DNA contamination was observed in preparations of AAV vector particles made using the CMV-cap construct (i.e., a cap expression vector not modified with a heterologous intron). As further described in Example 2, expression of Cap proteins was observed in cells transduced with standard AAV vector preparations by using a sensitive complementation assay. It is noted that traditional methods of AAV vector preparation typically utilize capsid expression cassettes that are small enough to be packaged within the AAV virion. Many copies of the cap plasmid are present within the cell following transfection, increasing the possibility for packaging. Alternative methods using cell lines with integrated copies of the rep and cap genes would most likely not circumvent this problem, as it has been shown that efficient expression of AAV from cell lines required amplification of the rep and cap genes out of their integrated form.

The expression cassette (10) of the present invention was designed to avoid cap gene packaging in AAV vector preparations, by introducing a heterologous intron sequence (30) into a cap expression cassette (10), wherein the heterologous intron sequence (30) is large enough to increase the total size of the expression cassette (10) to more than 5 kilobases.

It is known that the packaging size limit of AAV vectors is limited to the size of the parent wild-type AAV genome, which ranges in size based on the AAV serotype (i.e., from 4,087 to 4,767). See, e.g., Wu Z. et al., *Mol Ther* 18(1):80–86 (2010). For example, wild-type AAV-2 has a genome size of 4,679, and wild-type AAV-6 has a genome size of 4,683 (see TABLE 1). Therefore, in order to avoid packaging, the total size of the intron-modified expression cassette is at least 5 kilobases (kb), such as at least 5.2 kb, such as at least 5.5 kb, such as at least 6 kb, such as at least 6.5 kb, such as at least 7 kb, such as at least 7.5 kb, such as at least 8 kb, such as at least 9 kb, such as at least 10 kb, such as at least 50 kb, such as at least 100 kb or longer.

The heterologous intron sequence (30) included in the expression cassette (10) may comprise any heterologous intron sequence, provided that the heterologous intron sequence is operably linked to the transcriptional unit encoding the cap ORF (i.e., the expression cassette comprising the heterologous intron contains at least one splice donor and splice acceptor sites allowing for proper splicing of the transcription unit encoding the cap ORF). In accordance with this aspect of the invention, the total length of the intron-modified expression cassette (10) is at least 5 kb to avoid being packaged into AAV vector particles. Accordingly, the heterologous intron sequence (30) is chosen such that it comprises a sufficient length to bring the total size of the expression cassette (10) to a length of at least 5 kb. For example, as shown in FIGURE 2C, a representative embodiment of the expression cassette (10) comprising the AAV6 Cap ORF referred to as "pCMVE2Cap6", the total combined size of the promoter (20), Cap6 ORF (40), and polyA (50) is approximately 3.2 kb, therefore, the size of the heterologous intron (30) suitable for insertion in this exemplary expression cassette (10) would be at least 1.8 kb, in order to result in an expression cassette (10) having a total length of at least 5 kb.

In some embodiments, the expression cassette comprises multiple heterologous introns, wherein at least one of the heterologous introns is operably linked to the cap ORF, and wherein the multiple heterologous introns are chosen such that the combined

size of the heterologous introns brings the total size of the expression cassette to at least 5 kb.

Accordingly, in some embodiments, the at least one heterologous intron sequence, or the combined size of multiple heterologous intron sequences, is at least 1.8 kb, such as at least 2.0 kb, such as at least 2.5 kb, such as at least 3.0 kb, such as at least 3.5 kb, such as at least 4.0 kb, such as at least 4.5 kb, such as at least 5.0 kb, such as at least 6.0, such as at least 1 kb, such as at least 5 kb or longer.

In some embodiments, the heterologous intro (30) comprises at least one splice donor and one or more splice acceptor sites to allow for the proper splicing of the transcription unit encoding the cap OR (40), resulting in VP1, VP2, and VP3. A heterologous intron sequence can contain, or be modified to contain, appropriate splice donor and splice acceptor sites for the proper slicing of the transcription unit encoding the cap ORF based on the knowledge in the art regarding the wild type AAV genome. As known by those of skill in the art, the right side of a positive sense single strand wild type AAV genome encodes overlapping sequences of three capsid proteins, VP1, VP2 and VP3, which start from one promoter, designated p40. The molecular weights of these VP proteins are 87, 72 and 6 kiloDaltons, respectively (Jay, F.T., et al., *Proc. Natl. Acad. Sci.* 78:2927-31 (1981)). All three capsid proteins are translated from one mRNA. After this mRNA is synthesized, it can be spliced in two different manners: either a longer or shorter endogenous AAV intron can be excised, resulting in the formation of two pools of mRNAs: a 2.3 kb and a 2. kb long mRNA pool. Usually, and especially in the presence of adenovirus, the longer intron is preferred, so the 2. kb long mRNA represents the "major splice." In this form, the first AUG codon, from which the synthesis of VP1 protein starts, is cut out, resulting in a reduced overall level of VP1 protein synthesis. The first AUG codon that remains in the major splice is the initiation codon for VP3 protein. However, upstream of that codon in the same open reading frame (ORF) is an ACG sequence (threonine) which is surrounded by an optimal Kozak sequence, which contributes to a low level of synthesis of VP2 protein, which includes the amino acids of VP3 protein with additional N terminal residues. Jay, F.T., et al., *Proc. Natl. Acad. Sci.* 78:2927-31 (1981); Cassinotti, P., et al., *Virology* 167:176-84 (1988); Trempe, J.P., et al., *J Virology* 62(9):3356-63 (1988).

In another embodiment, as illustrated in FIGURE 2C, the heterologous intron (30) in the expression cassette (10) comprises a hybrid sequence with a first region (36)

comprising a heterologous intron sequence and a second region (38) comprising at least a portion of an AAV intron sequence (e.g., SEQ ID NO:3 which comprises a portion of the AAV-6 intron containing SA₁, SA₂). In accordance with such embodiments, the second region (38) comprising at least a portion of an AAV intron sequence may be from the same, or a different AAV serotype than the serotype of the cap ORF. The representative expression cassette shown in FIGURE 2C comprises AAV-6 Cap ORF (SEQ ID NO:1, encoding AAV-6 capsid protein, set forth as SEQ ID NO:2), and includes an hybrid intron (30) located between the CMVp and the cap ORF, wherein the hybrid intron comprises a first region (36) comprising an intron sequence from the heterologous gene EIF2S1 (SEQ ID NO:5), which contains a splice donor (SD) sequence, and a second region (38) comprising at least a portion of the endogenous AAV-6 intron (SEQ ID NO:4), which comprises a subregion (SEQ ID NO:3) containing the first endogenous splice acceptor site (SA₁) and the second endogenous splice acceptor site (SA₂) from AAV-6.

The heterologous intro (30) may be positioned in any suitable location within the expression cassette (10) provided that the intron is operably linked to the cap OR (40) to allow for appropriate expression of the capsid proteins (VP1, VP2 and VP3). For example, the heterologous intro (30) may be positioned within the cap coding region, or between the promoter and the cap coding region, or in the 3' untranslated region. In one embodiment, as illustrated in FIGURE 2C, the heterologous intro (30) is positioned between the promote (20) and the cap OR (40).

The intron-modified cap expression cassette (10) as described herein may be generated using standard molecular biology techniques and can be assessed for the ability to produce Cap proteins VP1, VP2 and VP3 using Western blot analysis, as described in Example 3. The expression cassette (10) can be further assessed for the ability to produce Cap proteins capable of packaging AAV vector particles using the methods described in Example 3.

The promote (20) for use in the expression cassette which is operably linked to the AAV cap ORF may be the promoter naturally associated with the AAV cap gene (i.e., p40), or may be a heterologous (non-AAV) promoter. Preferably, a strong promoter such as a cytomegalovirus (CMV) promoter, particularly the CMV major IE gene promoter, is used to express the AAV cap gene. Other examples of suitable strong promoters include, but are not limited to, the retroviral Rous sarcoma virus (RSV) LTR

promoter, SV40 promoter, the dihydrofolate reductase promoter, the beta-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1 promoter.

As illustrated in FIGURE 2C, in one embodiment, the expression cassette (10) comprises a promoter (20), an intron (30) comprising heterologous (non-AAV) sequences operably linked to the cap ORF (40), and a polyadenylation sequence (50). In one embodiment, the expression cassette (10) is provided in the form of pCMVE2Cap6 (SEQ ID NO:7). As illustrated in FIGURE 2C, the pCMVE2Cap6 construct contains an expression cassette comprising the cap ORF (40) driven by the CMVp (20) and an intron (30) comprising non-AAV sequences, wherein the total size of the expression cassette (10) is at least 5 kb to prevent packaging of the nucleic acid sequence encoding AAV Cap protein in the AAV vector particles. The representative expression cassette shown in FIGURE 2C, the AAV cap ORF (40) comprises AAV-6 Cap ORF (SEQ ID NO:1), and includes a hybrid intron (30) located between the CMVp and the cap ORF, wherein the hybrid intron comprises a first region (36) comprising an intron from the heterologous gene EIF2S1 (SEQ ID NO:5) containing a splice donor sequence, and a second region (38) comprising at least a portion of the endogenous AAV-6 intron (SEQ ID NO:4) which comprises a subregion (SEQ ID NO:3) containing the first endogenous splice acceptor site (SA₁) and the second endogenous splice acceptor site (SA₂) from AAV-6.

While the invention is described in connection with the representative AAV-6 cap ORF and intron sequences, it is contemplated that the compositions and methods may include sequences corresponding to the Cap ORF (e.g., SEQ ID NO:1, encoding SEQ ID NO:72) from AAV-6 and the intron region corresponding to the AAV-6 intron (SEQ ID NO:4), or at least a subregion thereof containing the SA₁ and SA₂ splice acceptor sites corresponding to the AAV-6 subregion (SEQ ID NO:3) obtained from other known AAV serotypes, such as, for example, the Cap ORF from AAV-2 (SEQ ID NO:8, encoding the AAV-2 capsid protein, set forth as SEQ ID NO:9). Capsid proteins suitable for use in the expression cassettes and methods of the invention include capsid proteins from AAV serotypes that are human or non-human. Representative AAV serotypes that may be included in the compositions and methods of the invention are provided in TABLE 1, and include Serotypes AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAV-8, AAV-9, AAV-10, AAV-11, AAV-12 and AAV-13.

TABLE 1: AAV Serotypes for Use in the Compositions and Methods

AAV Serotype	Genome Size	GenBank Ref Number (NCBI accessed 8/11/2010), wherein each sequence listed below is hereby incorporated by reference.
AAV-1	4,718 bp linear genomic	NC_002077.1
AAV-2	4,679 bp linear genomic	NC_001401.2
AAV-3	4,726 bp linear genomic	NC_001729.1
AAV-3B	4,722 bp linear genomic	AF028705.1
AAV-4	4,767 bp circular genomic	NC_001829.1
AAV-5	4,642 bp linear genomic	NC_006152.1
AAV-6	4,683 bp linear genomic	AF028704.1
AAV-7	4,721 bp linear genomic	NC_006260.1
AAV-8	4,393 bp linear genomic	NC_006261.1
AAV-9	4,385 bp linear genomic	AX753250.1
AAV-10	4,102 bp linear genomic	AY631965.1
AAV-11	4,087 bp linear genomic	AY631966.1
AAV-12	4,213 bp linear genomic	DQ813647.1
AAV-13	4,180 bp linear genomic	EU285562.1

In other embodiments, the expression cassette (10) may optionally include sequences encoding the AAV Rep 78/68 proteins and/or adenoviral helper functions (e.g., the adenoviral proteins E1, E2a, and E4 ORF6).

5 In some embodiments, the expression cassette (10) is included in a genetic element, such as a vector, which may be delivered to a host cell, e.g., naked DNA, a plasmid, phage, transposon, cosmid, episome, a protein in a non-viral delivery vehicle (e.g., a lipid-based carrier), virus, etc., which transfers the sequences carried thereon. The selected vector may be delivered by any suitable method, including transfection, electroporation, liposome delivery, membrane fusion techniques, high velocity
10 DNA-coated pellets, viral infection and protoplast fusion. In some embodiments, the expression cassette (10) is included in a vector, such as a plasmid, along with an origin of replication which allows for replication of the plasmid in an appropriate host cell. In some embodiments, the expression cassette (10) is included in a vector, such as a plasmid comprising nucleic acid sequences allowing for selection in a suitable host
15 (e.g., sequences that encode a phenotypic marker or reporter gene detectable by a suitable assay). In some embodiments, the expression cassette (10) is present in a host cell, either as an episome or stably integrated into the genome of the host cell.

In some embodiments, the intron-modified cap expression cassette is contained in a vector that optionally includes sequences encoding the AAV Rep 78/68 proteins and/or
20 adenoviral helper functions (e.g., the adenoviral proteins E1, E2a, and E4 ORF6). In some embodiments, the intron-modified cap expression cassette is contained in a vector that does not contain an AAV rep gene. In some embodiments, the intron-modified cap expression cassette is contained in a vector that contains the AAV rep gene in a separate transcriptional unit and optionally also contains the adenoviral proteins E1, E2a, and E4
25 ORF6.

In another aspect, the invention provides a method of reducing cap gene contamination in AAV vector particles. The method comprises: (a) introducing an AAV vector into a suitable host cell comprising an intron-modified cap expression cassette as described herein; (b) expressing rep and adeno helper functions in the host
30 cell; and (c) culturing the host cell to produce AAV vector particles.

The intron-modified cap expression cassette may be introduced into a host cell using standard DNA transfection techniques. See, e.g., Sambrook et al., *Molecular*

Cloning: A Laboratory Manual, 3d Ed., Cold Spring Harbor Press, Plainview, New York (2000).

The transforming DNA may or may not be integrated into the genome of the host cell. In one embodiment, the host cell is a mammalian packaging cell which also comprises rep and adeno helper functions sufficient for generating infectious AAV vector particles. In some embodiments, the host cell further comprises an AAV vector. As described herein, when a suitable AAV vector is introduced into a packaging cell, such as a cell comprising an intro-modified cap expression construct as described herein, the AAV vector can be assembled into a viral particle with reduced or no cap gene contamination.

In accordance with this aspect of the invention, the production of AAV vectors comprises the preparation of mammalian packaging cells that comprise an intron-modified cap expression cassette, a polynucleotide encoding an AAV rep gene and adenovirus helper functions (e.g., a polynucleotide encoding an adenovirus E4orf6 gene). In some embodiments, the AAV rep gene and the intron-modified cap expression cassette are provided on a single vector. In other embodiments, the rep and cap genes are provided on separate vectors. The packaging cells are then infected or transfected with a plasmid comprising the AAV inverted terminal repeat regions positioned on either side of a polynucleotide encoding a gene of interest. Under suitable conditions expression of the rep, cap, and E4orf6 genes of the packaging cell results in the synthesis of the Rep, Cap, and E4orf6 proteins which mediate replication and encapsidation of the AAV vector. Providing a polynucleotide which encodes a gene of interest between the AAV ITR sequences of the AAV vector results in packaging of the polynucleotide of interest into an infectious AAV vector particle which can be used to deliver the gene of interest to a desired host cell.

In some embodiments, the AAV vector particles generated in accordance with the methods of this aspect of the invention comprise a nucleic acid sequence encoding a heterologous (non-AAV) gene, such as a therapeutic gene. Typically, in the context of the present invention, such heterologous genes are located within the AAV vector, which vector is flanked by terminal repeat (ITR) regions and therefore can be replicated and encapsidated into AAV vector particles. Target heterologous genes can be used in this invention to generate AAV vectors for a number of different applications. Such heterologous genes include, but are not limited to, (i) polynucleotides encoding proteins

useful in other forms of gene therapy to relieve deficiencies caused by missing, defective or sub-optimal levels of a structural protein or enzyme; (ii) polynucleotides that are transcribed into anti-sense molecules; (iii) polynucleotides that are transcribed into decoys that bind transcription or translation factors; (iv) polynucleotides that encode cellular modulators, such as cytokines; (v) polynucleotides that can make recipient cells susceptible to specific drugs, such as the herpes virus thymidine kinase gene, and the like; and (vi) polynucleotides for the treatment of various cancers.

Methods of culturing packaging cells and exemplary conditions which promote the release of AAV vector particles, such as the producing of a cell lysate, may be carried out as described in Examples 1–4 herein. Producer cells are grown for a suitable period of time in order to promote release of virus into the media. Generally, cells may be grown for about 24 hours, about 36 hours, about 48 hours, about 72 hours, about 4 days, about 5 days, about 6 days, about 7 days, about 8 days, about 9 days, up to about 10 days. After about 10 days (or sooner, depending on the culture conditions and the particular producer cell used), the level of production generally decreases significantly. Generally, time of culture is measured from the point of viral production. For example, in the case of AAV, viral production generally begins upon supplying helper virus function in an appropriate producer cell as described herein. Generally, cells are harvested about 48 to about 100, preferably about 48 to about 96, preferably about 72 to about 96, preferably about 68 to about 72 hours after helper virus infection (or after viral production begins).

In some embodiments, the methods further comprise purification steps, such as treatment of the cell lysate with benzonase, purification of the cell lysate over a CsCl gradient, or purification of the cell lysate with the use of heparin sulphate chromatography, as described in Halbert C.L. and Miller, A.D., *Methods Mol Biol.* 246:201–212 (2004), hereby incorporated herein by reference.

The AAV vector particles produced using the captron plasmid have a reduced amount or an undetectable amount of cap gene as compared to AAV vector particles generated using standard cap6 packaging plasmids (non-intron modified), as described in Examples 2–4 herein. The presence or amount of cap DNA contamination in a preparation of AAV vector particles can be determined using quantitative PCR analysis, for example, as described in Example 2, and/or the presence or amount of cap gene contamination in a preparation of AAV vector particles can be determined by a complementation assay, as described in Example 3.

In another aspect, the present invention provides a method of reducing the immune response in a mammalian subject undergoing treatment with an AAV vector comprising administering to the mammalian subject a preparation of AAV vector particles comprising a nucleic acid sequence encoding a therapeutic molecule, wherein
5 the preparation of AAV vector particles was produced with an intron-modified cap expression cassette, wherein the immune response in the mammalian subject exposed to an AAV vector generated using the intron-modified cap expression cassette is significantly reduced or absent as compared to the immune response generated with a preparation of AAV vector particles generated using a standard cap expression cassette.

10 The AAV vector particles for use in the methods of this aspect of the invention, generated using the intron-modified cap expression cassette as described herein, avoid the generation of an immune response to the AAV capsid sequences in a mammalian subject, as demonstrated in Example 4 herein. The AAV vector particles are administered to a mammalian subject in sufficient amounts to provide sufficient levels of gene transfer and
15 expression to provide a therapeutic benefit without undue adverse effects, such as an immune response. In some embodiments, the use of the AAV vector particles generated using the intron-modified cap expression cassette obviates the need for treatment of the subject with immunosuppressive drugs, as described in Example 4, due to the reduction or absence of immune response in the subject to the AAV vector treatment.

20 The AAV vector particles may be administered to a mammalian subject using conventional and pharmaceutically acceptable routes of administration include, but are not limited to, direct delivery to a desired organ (e.g., the liver (optionally via the hepatic artery) or lung), oral, inhalation, intranasal, intratracheal, intraarterial, intraocular, intracochlear, intravenous, intramuscular, subcutaneous, intradermal, and other parental
25 routes of administration. Routes of administration may be combined, if desired.

Dosages of the AAV vector particles will depend primarily on factors such as the condition being treated, the age, weight and health of the patient, and may thus vary among patients. For example, a therapeutically effective human dosage of the AAV vector particles is generally in the range of from about 0.1 mL to about 100 mL of
30 solution containing concentrations of from about 10^9 to 10^{16} genomes of the virus vector. A preferred human dosage for delivery to large organs (e.g., liver, muscle, heart and lung) may be about 5×10^{10} to 5×10^{13} AAV genomes per 1 kg, at a volume of about 1 to 100 mL. A preferred dosage for delivery to eye or ear (cochlea) is about 5×10^9 to

5 5×10^{12} genome copies, at a volume of about 0.1 mL to 1 mL. The dosage will be adjusted to balance the therapeutic benefit against any side effects and such dosages may vary depending upon the therapeutic application for which the vector is employed. The levels of expression of the transgene can be monitored to determine the frequency of dosage resulting in viral vectors.

In another aspect, the present invention provides a complementation assay for measuring the presence or amount of cap or rep gene contamination in a preparation of recombinant AAV vector virions.

10 In one embodiment, the complementation assay is carried out to measure the presence or amount of cap gene contamination in a preparation of AAV vector virions and comprises (a) infecting a host cell with a test preparation of AAV vector, wherein the host cell comprises: (i) an indicator AAV vector with a detectable marker, (ii) a rep expression vector; and (iii) adenovirus helper functions; (b) culturing the host cell for a period of time sufficient to generate AAV vector virions from the indicator AAV vector;
15 (c) contacting an indicator cell with cell lysate obtained from the host cells cultured in accordance with step (b); and (d) determining the presence or amount of indicator cells that are positive for the detectable marker, wherein a positive result indicates that the test preparation of AAV vector virions contains cap gene contamination.

In another embodiment, the complementation assay is carried out to measure the presence or amount of rep gene contamination in a preparation of AAV vector virions and comprises (a) infecting a host cell with a test preparation of AAV vector, wherein the host cell comprises: (i) an indicator AAV vector with a detectable marker, (ii) a cap expression vector, and (iii) adenovirus helper functions; (b) culturing the host cell for a period of time sufficient to generate AAV vector virions from the indicator AAV vector;
20 (c) contacting an indicator cell with a cell lysate obtained from the host cells cultured in accordance with step (b); and (d) determining the presence or amount of indicator cells that are positive for the detectable marker, wherein a positive result indicates that the test preparation of AAV vector virions contains rep gene contamination.

FIGURE 1 provides a flow diagram illustrating a representative, non-limiting embodiment in accordance with this aspect of the invention.
30

In some embodiments, the complementation assay further comprises treating the test preparation of AAV vector and/or the cell lysate obtained from the host cells with benzonase, for example, as shown in FIGURE 1 and described in Example 3.

Indicator AAV vectors for use in the complementation assay comprise at least one detectable or selectable marker(s). Suitable markers include genes which confer antibiotic resistance or sensitivity, or impart color, or change the antigenic characteristics when cells which have been transduced with the AAV vector particles are grown in an appropriate selective medium. Particular selectable marker genes useful in the practice of the invention include the Alkaline phosphatase gene, Green fluorescent protein (GFP), Neomycin resistance gene (encoding Neomycin phosphotransferase) that allows selection in mammalian cells by conferring resistance to G418 (available from Sigma, St. Louis, Mo.) and the Hygromycin-B resistance gene (encoding Hygromycin-B-phosphotransferase) that confers resistance to Hygromycin-B. Other suitable markers are known to those of skill in the art.

The following examples merely illustrate the best mode now contemplated for practicing the invention, but should not be construed to limit the invention.

15

EXAMPLE 1

This Example demonstrates that genes without flanking AAV ITRs, such as those present in packaging constructs used for expression of Cap and Rep during AAV vector production, can be packaged into AAV vectors and expressed in cells transduced with the AAV vectors.

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Rationale:

In several clinical trials using AAV vector transduction in human subjects, an immune response to capsid has been detected. For example, in a clinical trial for hemophilia B, two of seven subjects given an AAV vector-expressing clotting factor IX (FIX) developed a transient self-limiting increase in liver transaminases, followed by clearance of the FIX-expressing cells at 4 to 8 weeks after delivery (Manno, C.S., et al., *Nat. Med.* 12:342-347 (2006)). In this clinical trial for hemophilia B, a cytotoxic lymphocyte (CTL) response to AAV capsid but not to FIX was detected in peripheral blood mononuclear cells. In another clinical trial involving lipoprotein lipase, clearance of AAV vector-transduced cells coincided with a CTL response towards the capsid, but not the transgene (Mingozzi, F., et al., *Blood* 114:2077-2086 (2009)). It is important to know how this immune response to AAV capsid is generated in order to prevent it. One hypothesis suggests that persistence of Cap proteins from the vector inoculum results in

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clearance of the transduced cells. An alternative hypothesis suggests that the immune response results from Cap expression that occurs in the transduced cells due to cap gene transfer by AAV vectors. A clinical protocol that uses transient immunosuppression may be successful if the former hypothesis is true, but if the later hypothesis is true, an immune response is likely to occur when immunosuppression is lifted. It is also possible that both mechanisms occur, and in this case, successful long-term transduction by AAV vectors will require both transient immunosuppression and methods to prevent AAV vector contamination with capsid-expressing DNA. The observation of a capsid-directed immune response even after 16 weeks of immunosuppression in a canine model of muscle gene transfer suggests a stable source of antigen (Wang, Z., et al., *Mol. Ther.* 15:1160-1166 (2007)). We also have observed an immune response to AAV6 capsid that significantly decreased transgene expression within 3 weeks after transduction in a canine model of lung gene transfer (Halbert, C.L., et al., *Mol. Ther.* 18:1165-1172 (2010)). In this canine model, immune suppression promoted long term gene expression (4 months) but that was lost after immune suppression was lifted. *Id.* Assuming that the capsid protein associated with vector virions had disappeared by this time, the delayed immune response suggests a continuous source of capsid expression, perhaps due to transfer of cap genes by the vector preparation.

The development of AAV vectors for clinical use is marked by continuing efforts to improve efficiency and to remove impurities. Early methods of AAV vector production resulted in contamination with replication-competent AAV. While several strategies were used to prevent such contamination (see, e.g., Allen, J.M., et al., Debelak, D.J., Reynolds, T.C., and Miller, A.D., *J. Virol.* 71:6816-6822 (1997); Cao, L., et al., *J. Virol.* 74:11456-11463 (2000); and Nony, P., et al., *J. Virol.* 77:776-781 (2003)), packaging of other DNA sequences into AAV virions can still occur. For example, DNA encoding the bacterial ampicillin resistance gene originating from plasmids used to make AAV vectors has been found in AAV vector preparations at 0.5-7% of the level of vector genomes (vg) (Chadeuf, G., et al., *Mol. Ther.* 12:744-753 (2005)). DNA encoding the ampicillin resistance gene was also found in tissues of mice, dogs, and non-human primates up to 5 months after AAV vector delivery, demonstrating that DNA impurities in AAV vector preparations can persist *in vivo*. *Id.* Furthermore, the presence of cap DNA sequences in clinical lots of AAV vectors at a level of 0.00018 cap DNA copies per vg has been reported by Hauck B., et al., *Mol. Ther.* 17:144-152 (2009)). However,

Hauck et al. did not detect capsid expression in recipient mice or cultured cells, as measured by quantitative PCR (qPCR) of reverse-transcribed mRNA obtained from the cultured cells.

This Example describes an experiment that was carried out to determine whether the immune response to Cap observed in the various clinical trials and animal models results from Cap expression that occurs in the transduced cells due to cap gene packaged in AAV vectors. This Example describes a comparison of the rate of AAV-mediated transduction of a gene that encodes human placental alkaline phosphatase (AP), either flanked by ITRs in the standard AAV vector configuration (designated pARAP4), or without any flanking AAV sequences (pRAP).

Methods:

Cell Culture: Human embryonic kidney (HEK) 293 cells (ATCC CRL 1573) and HTX cells, an approximately diploid subclone of human HT-1080 fibrosarcoma cells (ATCC CCL 121), were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% Cosmic Calf serum (Thermo Scientific) or 10% fetal bovine serum (FBS), respectively.

AAV vector plasmids.

ARAP4 is an AAV2-based vector (Allen, J.M., et al., *Mol. Ther.* 1:88-95 (2000)) which expresses Alkaline phosphatase (AP) and contains the RSV promoter/enhancers, the AP cDNA, and SV40 polyadenylation sequences, flanked by AAV2 ITRs.

The plasmid pRAP was generated by deleting the AAV2 ITRs from pARAP4.

The ACWRZn and ACZn vectors express a nuclear-localized β gal protein flanked by AAV ITRs (Halbert, C.L., et al., *Hum. Gene Ther.* 18:344-354 (2007)).

The ACAGhAAT vector expresses human α 1-antitrypsin (Halbert, C.L., et al., *Mol. Ther.* 18:1165-1172 (2010)).

The ACF3'B vector was derived from ARAP4 and contains the 3' portion of the human CFTR cDNA (2.1 kb region downstream of the HpaI site).

The ACMVcFIX vector expresses canine clotting factor FIX and was kindly provided by Katherine High (Children's Hospital of Philadelphia).

Helper Plasmids.

The plasmid pDGM6 encodes Rep, Cap and adenovirus helper functions (Gregorevic, P., et al., *Nat. Med.* 10:828-834 (2004)). pLadeno5 contains adenovirus

E2A, E4, and VA RNA regions to provide helper functions. pMTrep2, pMTrep6, and pCMVcap6 (Halbert, C.L., et al., *J. Virol.* 75:6615-6624 (2001)) were also used to supply packaging functions. All AAV vectors were made using the cap gene from AAV serotype 6.

- 5 The plasmid pDGM6Δcap was made by removing the Swal/BstZ171 cap fragment of pDGM6. The AAV vector plasmids were propagated in the bacterial strains Sure (Stratagene) or JC8111 (Boissy, R., and Astell, C.R., *Gene* 35:179-185 (1985)), and the packaging and helper plasmids were propagated in the DH5α strain of *E. coli*.

AAV vector production and characterization.

- 10 AAV-6 vectors were generated by cotransfection of vector and helper plasmids, as shown in Table 1, into 293 cells seeded at 4×10^6 cells per 10-cm dish the prior day. For the 2-plasmid protocol, the plasmid pDGM6 was co-transfected with the vector plasmids for generating the AAV vectors (20 μg and 10 μg per plate of 293 cells, respectively). For the 4-plasmid protocol, plasmids pLadeno5, pCMVcap6, pMTrep2 (or pMTrep6),
15 described in Halbert C.L. et al., *J. Virol* 75:6615-6625 (2001), hereby incorporated herein by reference, were used to supply helper and packaging functions (5 μg of each plasmid per plate of cells). Concentration and purification of AAV vectors was done as described in Halbert, C.L., and Miller, A.D., *Methods Mol. Biol.* 246:201-212 (2004)), hereby incorporated herein by reference, except that the centrifugation step in sucrose prior to
20 loading on to a heparin column was omitted.

- Southern blot analysis was carried out as described in Halbert, C.L., et al., *J. Virol.* 71:5932-594 (1997) to determine the number of vector genomes (vg) in the AAV vector preparations. It was determined that the AAV-6 vector preparations described in Examples 1-4 herein had generally between 10^{11} and 10^{12} vg per ml with a
25 vg to FFU ratio in HTX target cells of 2×10^4 to 5×10^4 for the AP-expressing vectors, and 10^5 for the βgal-expressing vector.

Quantitative PCR.

- Virus was prepared for qPCR by treating 10^{10} to 10^{11} genome-containing virions (determined by Southern analysis) with benzonase (100 units per reaction, 5 mM Tris pH
30 8.0, 10 μg/ml bovine serum albumin, 0.1 mM MgCl₂) in 500 μl volume for 1 h at 37°C. Next, AAV virion DNA was extracted by treatment with proteinase K (100 ng/ml) and 50 μl of 10X SET buffer (10% SDS, 1 M Tris pH 7.5, 0.05 M EDTA pH 8.0) for 1 h at 50°C, followed by two phenol/chloroform extractions, and precipitated with 5 μg tRNA

and 0.1 M NaCl in ethanol. Virion DNA pellets were resuspended in 100 µl distilled water.

A qPCR assay was carried out as follows: Maxima SYBR Green/ROX kit (Fermentas, Burlington, Canada) was used for the detection of rep and cap genes using the ABI 7900HT Real Time PCR System. Each reaction was done in triplicates with 25 µl volumes containing 12.5 µl of 2X master mix, 150 nM of each primer, and 1 µl template (resuspended vector DNA). The primers RepF (CGGGGTTTTACGAGATTGTG (SEQ ID NO:10), nucleotide positions 326-345) and RepR1 (CGCCATTTCTGGTCTTTGTG (SEQ ID NO:11), nucleotides 742-721) were used for amplification of rep yielding a 416 bp PCR product and primers CapF (CCACAAGAGCCAGACTCCTC (SEQ ID NO:12), nucleotides 2655-2674) and CapR540 (GCCATCATTCGTCGTGACC (SEQ ID NO:13), nucleotides 3193-3175) were used for cap gene amplification giving a 539 bp product. Cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 40 s. Plasmid pDGM6 representing 10^2 to 10^6 copies was used to generate the standard curve to calculate the copy number in the samples for both rep and cap (linearized with NotI for 1 of 2 cap reactions and both rep reactions).

Primers for canine FIX and for βgal were used to determine the amount of vector genomes in reaction. For cFIX, primers cFIXF1 (CCACTGTATTGAGCCTGATGTTAAA) (SEQ ID NO:14) and cFIXR2 (GCTCTGTATGTTCCCTCTTCTCGGT) (SEQ ID NO:15) were used, and pCMVcFIX was used for standardization. For βgal, primers lacZqF1 (GCGTAACTCGGCGTTTCAT) (SEQ ID NO:16) and lacZqR1 (GCGCTCAGGTCAAATTCAGAC) (SEQ ID NO:17) were used, and pACZn was used for standardization. All other AAV vectors used values previously determined by Southern analysis. When comparisons were available, vector genome values derived by Southern analysis and by qPCR varied by less than 2-fold.

Comparison of the rate of AAV-mediated transduction of a gene that encodes human placental alkaline phosphatase (AP), either flanked by ITRs in the standard AAV vector configuration (designated pARAP4), or without any flanking AAV sequences (pRAP).

In this experiment, we compared the rate of AAV-mediated transduction of a gene that encodes human placental alkaline phosphatase (AP), either flanked by AAV ITRs in

the standard AAV vector configuration (pARAP4), or without any flanking AAV sequences (pRAP). AAV virions were produced by cotransfection of each of these plasmids with a plasmid that expresses AAV Rep and Cap and adenovirus helper proteins (pDGM6) and the virions were purified by using heparin columns. HTX cells were then exposed to the purified AAV preparations and foci of AP-positive cells were quantitated.

Results:

Table 2 shows the results of AAV vector transduction in HTX cells of AAV vector virions containing an AP gene with or without flanking AAV terminal repeats (ITRs), measured in AP⁺ FFU produced in HTX cells exposed to the purified AAV preparations. The results shown in Table 2 are the means of duplicate determinations in a representative experiment.

Table 2. Gene transfer in HTX cells after exposure to AAV vector preparations made using the AP gene with and without flanking AAV terminal repeats^a

Transfected plasmids used to generate AAV vector preparations	AP ⁺ foci produced in HTX cells per transfected 10-cm dish:	
	before benzonase treatment	after benzonase treatment
pARAP4 + pDGM6	1.5×10^7	1.3×10^7
pRAP + pDGM6	7.5×10^2	3.6×10^2
pRAP + pUC18	0.25	0.25
pRAP + pUC18, pDGM6 + pUC18 ^b	Not done	0.30
pRAP + pACZn + pDGM6	Not done	1.8×10^3

^a 293 cells were transfected with the indicated plasmids one day after seeding the cells in 10-cm dishes. Three days after transfection, AAV virions were harvested and purified as described above. Purified virions were left untreated or were treated with benzonase to remove DNA that might be bound to the outside of virions. Virions capable of expressing AP were measured using HTX cells as targets for infection. Results are means of duplicate determinations in a representative experiment.

^b 293 cells in 10-cm dishes were independently transfected with pUC18 and pRAP, or pUC18 and pDGM6. After harvest of cells and medium, cell lysates were combined, treated with benzonase, co-purified on a heparin column, and then assayed for AP⁺ FFU.

As shown in Table 2, AAV vector made with the plasmid that does not contain AAV sequences (pRAP) did indeed induce AP⁺ foci in HTX cells (Table 2, row 2). However, the number of AP⁺ foci produced by AAV virus made with the pRAP plasmid was 4 to 5 orders of magnitude lower than that of the standard vector plasmid pARAP4 (Table 2, row 1), which contains the AP gene flanked by AAV ITRs. AAV vector preparations made with a control plasmid (pUC18) instead of the plasmid encoding AAV Rep and Cap proteins (pDGM6) exhibited low levels of background AP⁺ foci (see Table 2, row 3), as expected.

To test the possibility that the pRAP DNA was transferred to HTX cells by binding to the surface of the AAV virions, AAV vector preparations were treated with benzonase to destroy DNA outside of the virions and were repurified on heparin columns. As further shown in Table 2, benzonase treatment did not destroy the ability of virions made with pRAP to transduce target cells, indicating that the AP gene without the ITR sequences was packaged within the AAV virions (see Table 2, columns 2 and 3).

To further rule out transfer of pRAP DNA to HTX cells via an interaction with the surface of the capsid, two separate cell lysates were made, one from 293 cells transfected with only the non-ITR pRAP plasmid and one from cells transfected with only the helper plasmid expressing the AAV capsid proteins. The two preparations were combined, treated with benzonase and purified. As shown in Table 2, infection of HTX cells with the combined preparation yielded only low background levels of AP, showing that the transfer of AP that we observed in the HTX cells could not be explained by interactions between DNA and fully formed particles (see Table 2, row 4).

We next tested whether transfer of the AP gene from AAV vector generated using pRAP (non-ITRs) would still occur in competition with a genuine AAV vector (containing ITRs), to address the possibility of transfer of genes without AAV ITR sequences during routine AAV vector production. For this experiment, we used the AAV vector ACZn that expresses a nuclear-localized β -galactosidase (β gal) flanked by ITRs to allow independent quantitation of gene transfer by pRAP and the AAV vector ACZn. As shown in Table 2, indeed, the AP gene was transferred by virus produced by cotransfection of pRAP, pACZn and pDGM6 (see Table 12, row 5). The rate of AP gene transfer was 5-fold higher in the presence of the ACZn AAV vector than in its absence, while the titer of the ACZn vector produced in the presence of pRAP was similar to that of other preparations made without it (2.5×10^5 β gal⁺ focus-forming units (FFU) per

10-cm dish), data not shown. These results demonstrate that genes without flanking AAV ITRs are packaged in AAV vector virions and transferred and expressed in cells infected by AAV vector virions.

5 **Conclusion:**

In summary, the above experiments confirm that DNA sequences without flanking AAV ITRs can be packaged into AAV virions, and in this case, are transferred and expressed at a rate that is 4 to 5 orders of magnitude lower than that of a typical AAV vector that contains DNA that is flanked by ITRs.

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EXAMPLE 2

This Example demonstrates that DNA encoding Rep and Cap originating from helper plasmids that do not contain flanking ITR sequences are packaged into AAV vector virions and expressed in cells infected with such virions.

15 **Rationale:**

The results described in Example 1 suggest that genes without flanking AAV ITRs, such as plasmids used for expression of Cap and Rep during AAV vector production, could be packaged into AAV vector virions and expressed in cells infected with such virions. To further address this issue, we first tested for the presence of cap and rep DNA in several AAV vector preparations by qPCR. We then developed a Cap and Rep complementation assay to determine whether the packaged cap and rep DNAs were expressed in infected cells, as described below.

20

1. qPCR Analysis for the Presence of cap and rep DNA in several AAV vector Preparations

25 **Methods:**

AAV vector preparations were made using the AAV vectors and helper plasmids as indicated in Table 3, using the methods described in Example 1. The AAV preparations were generated using either the single plasmid pDGM6 or using a combination of the plasmids pMTrep2, pCMVcap6, and pLadeno5, as described in Example 1. The plasmid pDGM6 carries rep, cap and adenovirus helper genes, while pLadeno5 carries only the adenovirus help genes. The genes carried by the AAV vectors are as follows: ACWRZn, LacZ; ARAP4, alkaline phosphatase; ACAGhAAT, human alpha1-antitrypsin; ACF3'B, the 3' half of the CFTR coding region.

30

Virus was prepared for qPCR by treating 10^{10} to 10^{11} genome-containing particles (determined by Southern analysis) with benzonase as described in Example 1. Next, virion DNA was extracted and was subjected to qPCR as described in Example 1.

Results:

- 5 Table 3 shows the quantitation of rep and cap DNA present in AAV vector virions as measured by qPCR analysis. The results shown in Table 3 are expressed as copies of DNA per 10^6 vector genomes (vg) as determined by Southern blot analysis. For the ACWRZn vector, the genome number was checked by qPCR after benzonase treatment and purification of virions, and was 63% of the value determined by Southern analysis.
- 10 Results are means of two experiments done in triplicate. The primers used to detect cap and rep DNA, are described in Example 1, and amplify products of 539 and 416 base pairs, respectively.

Table 3. Quantitation of rep and cap DNA in AAV vector virions by qPCR ^a

AAV vector	Adenovirus helper plasmid	rep and cap plasmids	Copies of cap per 10^6 vg	Copies of rep per 10^6 vg
ACWRZn	pLadeno5	pMTrep2, pCMVcap6	170	150
ARAP4 prep 1	pLadeno5	pMTrep2, pCMVcap6	58	70
ARAP4 prep 2	pLadeno5	pMTrep2, pCMVcap6	14	16
ARAP4 prep 3	pLadeno5	pMTrep2, pCMVcap6	36	32
ARAP4 prep 4	pDGM6	pDGM6	32	38
ACAGhAAT prep 1	pDGM6	pDGM6	26	27
ACAGhAAT prep 2	pDGM6	pDGM6	49	63
ACF3'B	pDGM6	pDGM6	7.0	9.1

- 15 ^a Vector preparations were made by transfections of HEK 293 cells with the indicated vectors and separate plasmids encoding Rep, Cap, and adenovirus helper functions, or with the indicated vectors and a single plasmid, pDGM6, encoding Rep, Cap and adenovirus helper functions. Virus was prepared for qPCR by treating 10^{10} to 10^{11} genome-containing particles (determined by Southern analysis) with benzonase. Next, virion DNA was extracted as described in Example 1 and was subjected to qPCR that amplifies a 539 base
- 20 pair region of the cap gene or a 416 base pair region of the rep gene. Results are expressed as copies of DNA per 10^6 vector genomes as determined by Southern analysis.

As shown in Table 3, the qPCR results showed that vector virions contained 7 to 170 copies of cap DNA and 9 to 150 copies of rep DNA were packaged for every million vector genomes (i.e., at levels of 0.0007% to 0.017% of the level of vector DNA). The rate of both cap and rep DNA packaging was similar for each vector preparation tested, and correlated inversely with the AAV vector yield; i.e., AAV vectors that routinely had higher production yields (e.g., ACF3'B) showed less rep and cap DNA packaging, while those AAV vectors with lower yields (e.g., ACWRZN) had more rep and cap DNA packaging.

These results are consistent with a model in which the rate of cap and rep DNA packaging is constant and independent of AAV vector packaging, and that normalization of cap and rep copy number to the vector copy number generates this inverse relationship. It is noted that the AAV vector preparations tested were made using the single plasmid pDGM6 or using plasmids pMTrep2, pCMVcap6, and pLaden05, and we found cap and rep DNA in virions made with either plasmid combination. One vector (ARAP4) was made with both methods, and we did not observe a difference in packaging of cap or rep DNA due to the helper plasmids used.

2. Development of a Sensitive Cap and Rep Complementation Assay to determine if Packaged Cap DNA is Expressed in Cells infected with AAV vectors

Methods:

Complementation Assay:

To determine whether the packaged cap and rep DNAs are expressed in vector infected cells, we developed a Cap and Rep complementation assay, as illustrated in FIGURE 1. In this assay, purified, benzonase treated AAV vector preparations (ACF3'B and ACAGhAAT) were made as described in Example 1. 293 cells were plated at 5×10^6 cells per 10-cm dish (day 0). The 293 cells were then infected on Day 1 with the purified benzonase-treated AAV vector preparations for 2 h and then the media was replaced to remove benzonase and residual vector. Soon afterwards, the cells were transfected with the plasmid combinations shown below in Table 4 that included various combinations of an AP-expressing AAV vector plasmid (pARAP4) and the plasmids used for AAV vector production. As a positive control, the AAV vector and all three AAV production plasmids were used, while remaining combinations lacked one or more plasmids. On day 2, the transfection/infection medium was aspirated and replaced with

medium with 2 mM added L-glutamine. On day 4, cells and medium were harvested, freeze/thawed 3 times, centrifuged to remove cell debris, and was frozen in 1 ml aliquots. Prior to infection of HTX cells, 1 ml of cell lysate was thawed and treated for 2 h with benzonase. Then the transducing titer of each cell lysate was determined by quantitating AP⁺ FFU on HTX cells. HTX cells were plated at 5×10^4 cells per well in 6-well dishes on day 0. The following day, medium was aspirated, cells rinsed once with PBS containing calcium and magnesium, then exposed to 500 μ l of cell lysate with 500 μ l of DMEM for 2 h, then 1 ml of DMEM containing 20% FBS was added. The cells were fixed and stained for AP expression 3 days later. Production of AP⁺ HTX cells using plasmid combinations lacking cap and/or rep DNA would indicate provision of these functions by the AAV vector stock used initially to infect the 293 cells.

Results:

Several AAV vectors, including ACF3'B and ACAGhAAT, were examined using the complementation assay and the results are shown in Table 4. The results shown in Table 4 are means of duplicate determinations.

Table 4. Results of Complementation Assay: Transfer of Cap and Rep function following infection with AAV vectors^a

			AP FFU per ml for plasmid combination:				
			1	2	3	4	5
			pLadeno5	+	+	+	+
			pMTrep6	+	-	-	-
			pCMVcap6	-	+	-	-
Expt	AAV vector	Amount (vg)	pARAP4	+	+	+	-
1	ACF3'B (AAV6)	2×10^{12}	ND	1.0×10^4	ND	7	3
1		none	ND	36	ND	ND	ND
2	ACAGhAAT (AAV6)	7×10^{10}	ND	4.5×10^3	ND	4	6
2		none	1.2×10^6	66	17	ND	ND
3	ACAGhAAT (AAV6)	7×10^{10}	ND	7.1×10^3	8	4	6
3		none	ND	89	ND	ND	ND
4	ACWRZn (AAV6)	7×10^{10}	3.6×10^5	3.1×10^4	3.9×10^3	ND	ND
4	ACWRZn (AAV2)	7×10^{10}	4.4×10^5	1.4×10^4	8	5	ND
4		none	5.5×10^5	<1	2	ND	ND

^aCap and Rep complementation assays were done as shown in Figure 1 and described above, except that the crude cell lysate was filtered (0.2 μ m-pore-size) to reduce background AP in experiment 4. Results are means of duplicate determinations.

5 Transfection by the complete set of plasmids resulted in the production of ARAP4 vector virus (plasmid combination #1). Importantly, infection with the ACF3'B AAV vector preparation followed by transfection with plasmid combination #2 (that contained vector, replication and helper functions but lacked a capsid-expressing plasmid), yielded 10^4 AP⁺ FFU per ml of lysate, indicating transfer of functional cap
10 genes by the ACF3'B vector. Only background levels of AP⁺ foci were observed with plasmid combinations #4 or #5.

As further shown in Table 4, the complementation assay also detected transfer of cap gene function by the second vector preparation, ACAGhAAT. In two experiments, using 7×10^{10} vg ACAGhAAT for each infection, an average vector titer of 5.8×10^3
15 AP⁺ FFU per ml was observed after transfection of plasmid combination #2 (that lacks the Cap expression plasmid). No production of AP vector was seen from transfection combination #3 that lacked rep, showing that rep function was not transferred by the ACAGhAAT vector.

Note that when no AAV vector preparation was added in the above experiments,
20 the plasmid combination that did not contain the cap construct produced 36, 66 and 89 AP⁺ FFU per ml (Table 4, experiments 1–3, plasmid combination #2). This was higher than the background AP values observed in combinations 3, 4 and 5, and we hypothesize that this is due to AP protein transfer (pseudotransduction). The Rep protein in this combination likely caused replication of the ARAP4 vector DNA resulting in higher levels of AP protein in the
25 lysate added to the target cells.

To test whether cap function could be transferred by an AAV vector with a different AAV serotype, we tested for transfer of cap function by an AAV vector packaged with AAV6 or AAV2 capsid proteins, and found vectors with both serotypes transferred cap gene function (Table 4, Exp. 4). In one case we also detected transfer of rep gene function,
30 indicating that both the cap and rep genes can be transferred and expressed, as might have been expected based on the presence of both cap and rep gene sequences in AAV virions (Table 3). We note that in this experiment, we filtered the virus produced by the

transfected 293 cells in an attempt to reduce transfer of AP protein (pseudotransduction). Indeed, this substantially reduced the background AP⁺ foci obtained for plasmid combination 2 when no AAV vector was used.

Conclusion:

- 5 The results described in this Example demonstrate that AAV vector preparations contain cap DNA, which can be expressed in infected cells, and may serve as an immunogen in animals.

EXAMPLE 3

- 10 This Example demonstrates that the insertion of a large intron into the cap gene of a packaging plasmid to generate a capsid expression cassette (cap + intron = "captron") that is too large for packaging into AAV virions is effective to eliminate transfer of capsid-expressing DNA in AAV vector transduced cells.

Rationale:

- 15 One feature of AAV vectors, often considered to be a limitation, is their small packaging capacity (limited to packaging DNA in the size range below about 4.7 to 5 kb). The inventors have exploited this size limitation in a strategy to minimize the packaging of functional cap genes in which expression of capsid in transduced cells is limited by introducing an intron into the capsid expression cassette (e.g., between the cap gene and
20 the promoter) such that the expression cassette would be too large to package into AAV virions. This Example describes the generation of a captron construct in which a large intron (4.3 kb) from the 5' untranslated region of the human EIF2S1 gene was inserted between the CMV promoter and the alternatively spliced intron in the AAV cap gene.

Methods:

- 25 ***1. Generation of intron-containing cap expression constructs.***

- A large intron (4.3 kb) from the 5' untranslated region of the human *EIF2S1* gene was inserted between the CMV promoter and the alternatively spliced intron in the AAV cap gene to make the plasmid pCMVEcap6 (FIGURE 2a). The 4,460 bp region including the 5' untranslated region intron from the human *EIF2S1* gene was cloned by PCR
30 amplification of DNA from the HTX cell line using primers EIF2S1inFN (GGCTAGCgcggtggagtgcgaag) (SEQ ID NO:18) and EIF2S1inRN (GGCTAGCttctgcaatttaacaaaag) (SEQ ID NO:19) and inserted into the NheI site in pCMVcap6 to generate pCMVEcap6. Unfortunately, this plasmid did not express

functional capsid protein, as determined by a Western blot analysis (FIGURE 2b), and further investigation using northern analysis and RT-PCR revealed that the *EIF2S1* intron prevented proper excision of the native AAV intron (data not shown).

A new construct with a hybrid *EIF2S1*/AAV intron was generated by removing a
5 200 bp fragment containing the *EIF2S1* splice acceptor and the AAV splice donor to make the plasmid pCMVE2cap6 (SEQ ID NO:7), illustrated in FIGURE 2C. The pCMVE2cap6 construct was generated by removal of a 200 bp KpnI fragment of pCMVEcap6 containing the splice acceptor from the *EIF2S1* intron and the splice donor of the AAV cap gene. The size of this gene from promoter to the end of the cap ORF is
10 about 7.5 kb, much larger than the 4.7 kb AAV packaging limit. Western blot analysis shown in FIGURE 2B, was carried out on cell lysates of 293T cells transfected with 10 µg DNA using anti-AAV VP1+VP2+VP3 mouse monoclonal antibody (American Research Products). 10 ul of lysate was loaded per lane.

As shown in FIGURE 2b, the pCMVE2cap6 construct expressed all three capsid
15 forms, verifying proper alternative splicing at the AAV splice acceptors.

2. Titer of AAV preparations made using the intron-containing cap constructs (Captron)

The titer of AAV preparations made using the intron-containing cap constructs were compared as follows. Crude AAV lysates were collected three days after
20 transfection of 293T cells with 4 µg pARAP4, 4 µg pDGM6Δcap and 2 µg of a cap plasmid (pCMVcap6, pCMVEcap6 or pCMVE2cap6). Cells and medium were freeze/thawed three times, centrifuged at 1,000 x g for 10 minutes to remove cells and debris, and filtered (0.2 µm-pore-size). ARAP4 titers from one experiment were determined by infecting HTX cells and staining for AP+ foci after three days. As shown
25 in Table 4, only a minor change was observed in yield between pCMVE2cap6 and pCMVcap6, even though the 2 µg of pCMVE2cap6 transfected contains about half as many copies as that of the 2 µg of pCMVcap6.

Table 5: ARAP4 vector titer

cap plasmid used	AP+ FFU/ml
pCMVcap6	3.0×10^6
pCMVEcap6	1.5×10^4
pCMVE2cap6	2.0×10^6
pUC19 control	40

3. Measuring Rep and/or Cap Gene contamination by the Complementation Assay

5 The Cap and/or Rep complementation assay, as described in Example 2, was used to test the effect of the intron on capsid expression by comparing parallel AAV productions made with pCMVcap6 and the captron plasmid, pCMVE2cap6. The results are shown in Table 6.

10 Table 6. Inclusion of a large intron in the cap gene (AAV captron vector) prevents transfer of Cap function in transduced cells, as determined by the complementation assay^a

AAV vector used for preinfection	cap plasmid used for vector production	Vector Amount (vg)	ARAP4 vector production (AP ⁺ FFU per ml) after transfection with plasmid(s) combination:			
			1	2	3	4
			pARAP4 pCMVcap6 pMTrep6 pLadeno5	pARAP4 - pMTrep6 pLadeno5	pARAP4 pCMVcap6 - pLadeno5	pARAP4 - - pLadeno5
ACZn	CMVcap6	7×10^{10}	9×10^4	5.3×10^2	0	0
ACZn	Captron (pCMVE2cap6)	7×10^{10}	1×10^5	2	1	1
None	None	None	6×10^4	0	0	0
ACMVcFIX	CMVcap6	7×10^{10}	1.6×10^5	2.8×10^3	3	3
ACMVcFIX	Captron (pCMVE2cap6)	7×10^{10}	1.9×10^5	3	1	3
None	None	None	2.2×10^5	2	3	1
ACF3'B	CMVcap6	3.8×10^1	ND	41	0	0
ACF3'B	Captron (pCMVE2cap6)	3.8×10^1	ND	0	3	3

^a HEK 293 cells were plated at 5×10^6 cells per 10-cm dish on day 1. On day 2, cells were preinfected with the indicated amounts of purified AAV vectors made using either the standard cap plasmid

(pCMVcap6) or the captron (intron-containing) cap plasmid (pCMVE2cap6), or were left uninfected. All vector preparations were treated with benzonase before use to remove non-encapsidated DNA. The virus and medium was removed from the cells two hours later and cells were re-fed with fresh media. One hour later the cells were transfected with plasmid combinations as indicated. pUC13 insert plasmid DNA was
 5 used to replace the DNA of any missing plasmid. On day 3, the medium was aspirated and cells were re-fed. On day 5, cells and medium were harvested (10 ml), freeze/thawed 3 times, centrifuged, and filtered to remove cell debris. Next, the cell lysate was treated with benzonase to remove extraneous DNA. The number of functional ARAP4 virions in the cell lysates were then determined by measuring AP+ focus-forming units (FFU) using HT-1080 human fibrosarcoma cells as targets for transduction. Results
 10 are means from one experiment done in duplicate (ACZn and ACF3'B) and means from two, five or four experiments done in duplicate (ACMVcFIX made with the CMVcap6 plasmid, the captron plasmid or none, respectively). Genes carried by AAV vectors; ACZn, LacZ; ACMVcFIX, canine clotting factor IX, ACF3'B, the 3' half of the CFTR coding region.

15 As shown in Table 6, transfection of cells with all 4 plasmids produced lysates with similar titers regardless of whether a standard vector, a captron vector, or no AAV vector was used to infect the cells (Table 6, combination 1), showing that prior infection of 293 cells with an AAV vector did not inhibit production of AAV virions. No transfer of AP was seen without rep (Table 6, combination 3), or without rep and cap
 20 (Table 6, combination 4). Cells infected with ACZn and ACMVcFIX AAV preparations made using the original pCMVcap6 did demonstrate expression of Cap protein by production of ARAP4 AAV vector (Table 6, combination 2). In contrast, cells infected with AAV preparations made using the captron plasmid did not produce Cap protein, as only a very low, background level of AP⁺ cells was observed (Table 6, combination 2).
 25 The third vector, ACF3'B, routinely had at least a 10-fold higher vector yield than other vectors, and therefore a lower cap to vg ratio. When 7×10^{10} vg was used for infection, no complementation was observed (data not shown). When 5-fold more vector was used, ACF3'B made with pCMVcap6 showed a detectible level of cap complementation while the AAV preparation made with the captron construct still exhibited no detectible transfer
 30 of cap function (Table 6). In summary, inclusion of a large intron sequence in the cap construct reduced Cap expression from all AAV vectors tested to undetectable levels as measured by this highly sensitive complementation assay.

4. *qPCR Analysis of rep and cap DNA present in AAV vector Preparations*

Presumably, fragments of the DNA encoding the cap gene are still packaged into AAV vector virions, but not in large enough sizes to allow cap gene transfer and expression. This hypothesis was confirmed by measuring cap and rep DNA via qPCR analysis in several vector preparations made with either the standard pCMVcap6 plasmid or the captron plasmid. Analysis of DNA from AAV vector virions by qPCR showed that both cap and rep DNA sequences were still packaged (Table 7). However, there was a trend toward reduced packaging of cap in vector preparations with the captron construct. The amount of packaged rep DNA was not affected by use of the captron plasmid. For each of the three captron vectors investigated, copies of cap were 1.5-2 fold lower than rep copies, whereas no significant difference in cap and rep packaging was observed in vectors made with pCMVcap6.

Table 7. Quantitation of rep and cap DNA sequences in AAV virion preparations made with the standard cap or the captron cap plasmids

AAV vector	cap plasmid used during production	Copies of cap per 10 ⁶ vg	Copies of rep per 10 ⁶ vg
ACZn	CMVcap6	240	240
ACZn	Captron	54	110
ACF3'B	CMVcap6	4.1	9.5
ACF3'B	Captron	9.8	15
ACMVcFIX	CMVcap6	140	150
ACMVcFIX	Captron	55	110

^a Virion DNA was prepared for qPCR as described for Table 3 and in Example 2. Vector genomes were quantitated by Southern analysis for the ACF3'B vector and by qPCR for the ACZn and ACMVcFIX vectors by using primers for βgal or cFIX, respectively. Results are means of two experiments done in triplicate. Results for ACMVcFIX are means of experiments using two different vector preparations.

5. *qPCR Analysis to detect cap mRNA in AAV vector-exposed cells*

We next used a qRT-PCR assay to detect cap mRNA in virus-exposed cells and to confirm the cap complementation assay results by using a second method.

Methods:

HTX cells were infected with an AAV6 vector (ACMVcFIX) made with either pCMVcap6 plasmid or the captron plasmid pCMVE2cap6 at an MOI of 4×10^6 vg per cell. To maximize sensitivity, HTX cells were infected at a multiplicity of infection (MOI) of 4×10^6 vg of ACMVcFIX vector per cell and 300 ng of total RNA from virus-exposed cells was used per qRT-PCR reaction. Total RNA was extracted using TRIZOL (Invitrogen) after three days. The RNA was treated with DNase and cDNA was synthesized using an oligo (dT₂₀) primer using SuperScript III Reverse Transcriptase (Invitrogen). qRT-PCR was conducted in triplicate using Maxima SYBR Green qPCR master mix (Fermentas) with cDNA from 300 ng of RNA. The primers and standards for cap and cFIX were the same as those used for the above qPCR analysis of virion DNA, except that both pDGM6 and pCMVcFIX standards were linearized for all reactions (with NotI and HindIII, respectively). All standard curve reactions included cDNA made from uninfected HTX cells in addition to the known amounts of linearized plasmid. All primers were used at 0.2 μ M and cycle conditions for both reactions were 95°C for 10 min, with 45 cycles of 95°C for 15 s and 60°C for 1 min, followed by a dissociation curve.

Results:

No cap mRNA could be detected from cells infected with the vector made with the captron construct in any of the triplicate reactions. However, in qRT-PCR reactions done on two mRNA samples from independent infections with AAV made using pCMVcap6, approximately one copy per reaction was detected in one of the triplicate reactions (2/6 positive reactions, with the correct size amplicon confirmed on an agarose gel). Even after 45 cycles, no amplification products were observed in negative controls (300 ng of mRNA from uninfected HTX cells), or in controls performed without RT (to confirm the absence of DNA contamination). These data show that cap mRNA transcribed from cap gene impurities in AAV vectors can be detected by qRT-PCR, and indicate that use of the captron plasmid for vector production eliminated expression of cap mRNA. The level of cFIX mRNA detected in these samples was 4.9×10^5 copies per reaction, suggesting that cap mRNA is made at a level 10^6 -fold lower than that of the vector transgene. This was at the limit of detection of qRT-PCR, and therefore the result cannot be accurately quantified. However, the result was reproducible and agrees with results from the complementation assay. Thus, data from both the complementation

assay and qRT-PCR assays confirm that use of this intron-containing construct eliminated capsid expression from AAV vectors.

Discussion of Examples 1-3

The results described in Examples 1-3 confirm previous reports of contamination of AAV vector preparations with DNA from plasmids lacking AAV sequences, as well as DNA from cap and rep plasmids used to make AAV vectors. However, in contrast to previous results (Hauck, B., et al., *Mol. Ther.* 17:144-152 (2009)), we show that cap DNA present in AAV vector preparations can express cap mRNA and protein in vector-transduced cells. To detect Cap protein expression, we used a sensitive complementation assay involving infection of AAV vector preparations followed by transfection of various plasmid combinations, and for RNA detection we exposed cells to more vector MOI (4×10^6 vg per cell) and analyzed more cell RNA (300 ng) than previously used (10^5 vg per cell and 200 ng RNA) in Hauck (2009).

Even though the amount of packaged cap DNA is orders of magnitude less than the vector DNA present in AAV vector preparations produced using standard cap plasmids (i.e., non-intron containing cap constructs), there may be significant levels of expressed capsid *in vivo* resulting from infection with standard AAV vector preparations due to the high number of AAV vectors required for therapeutic effect. For example, in the FIX clinical trial in which a capsid-specific response was observed, a dose of up to 2×10^{12} vg/kg was used (Manno, C.S., et al., *Nat. Med.* 12:342-347 (2006)). Thus, our results described in Examples 1-3 herein demonstrate that capsid protein made from cap DNA in AAV preparations are likely to be at least partially responsible for the capsid-directed immune response observed in canine and human clinical trials.

Based on data from canine studies showing a capsid-directed immune response following transient immunosuppression, a significant amount of capsid particles would need to persist for greater than 16 weeks *in vivo* for the response to be entirely due to administered capsid protein. Indeed, intact AAV virions have been detected up to 6 years after AAV gene transfer in the retina of dogs and primates (Stieger, K., et al., *Mol. Ther.* 17:516-523 (2009)). The authors in Stieger et al. (2009) hypothesized that the virions were from the initial inoculum, but our data suggest that virions also can be made from newly synthesized capsid proteins, which we have shown can be made in the absence of adenovirus helper functions (Allen J. M. et al., *Mol Ther* 1:88-95 (2000)).

While investigating strategies to prevent packaging of cap DNA, we noted that traditional methods of AAV vector preparation used capsid expression cassettes small enough to fit within the AAV virion. Many copies of the cap gene are present within the cell following transfection, increasing the possibility for packaging. Alternative methods
5 using cell lines with integrated copies of rep and cap would most likely not circumvent this problem, as it has been shown that efficient expression of AAV from cell lines required amplification of the rep and cap genes out of their integrated form. Contamination of AAV produced from a stable cell line containing ampicillin resistance gene DNA has already demonstrated that packaging of amplified DNA occurs (Chadeuf,
10 G., et al., *Mol. Ther.* 12:744-753 (2005); Chadeuf, G., et al., *J. Gene Med.* 2:260-268 (2000); and Liu, X., et al., *Mol. Ther.* 2:394-403 (2000)). Much work with AAV has also focused on purification methods, such as CsCl centrifugation protocols (Ayuso, E., et al., *Gene Ther.* 17:503-510 (2010)). These processes can remove DNA that is not packaged in virions and can separate empty particles from DNA-containing particles, which does
15 reduce the amount of injected capsid protein, but it is important to note that particles containing cap DNA are not empty, and are not likely to be completely excluded by any such process.

It has been reported that AAV vectors are able to express large genes by recombination of multiple gene fragments, but this process is relatively inefficient for
20 oversized constructs (Duan, D., et al., *Mol. Ther.* 4:383-391 (2001); Halbert, C.L., et al., *Nat. Biotechnol.* 20:697-701 (2002); Dong, B., et al., *Mol. Ther.* 18:87-92 (2010); Lai, Y., et al., *Mol. Ther.* 18:75-79 (2010); and Wu, Z., et al., *Mol. Ther.* 18:80-86 (2010)).

It is also unknown whether the cap DNA fragments recombine at the same rate, or whether vector recombination is due in part to the presence of the AAV ITRs. The
25 requirement of at least two fragments for recombination and the greatly reduced packaging rate of cap gene fragments in comparison to that of AAV vectors argues that recombination events leading to Cap expression should be exceedingly rare. Indeed, we observe no evidence of any capsid expression in cultured cells when using the captron construct. We conclude that AAV made with a large intron-containing cap gene offers a
30 less immunogenic alternative to vectors made with current capsid constructs and may promote longer-term transgene expression in animals and humans compared to previous vectors.

In addition, as would be understood by those of skill in the art, it is expected that the same strategy of introducing one or more introns into a helper plasmid or into a gene expression construct present in an AAV packaging cell, can be utilized to effectively reduce and/or eliminate the packaging of any undesired DNA that is present during AAV virus production into AAV virions, such as DNA encoding the AAV rep gene, a selectable gene such as the gene encoding ampicillin resistance, etc.

EXAMPLE 4

This Example demonstrates that there is a dramatic reduction in the immune response in dogs after delivery to muscle of a AAV6 vector encoding canine factor IX produced using the captron plasmid in comparison to the immune response observed after infection with an AAV6 vector encoding the same transgene produced using a standard cap plasmid.

Rationale:

Current studies indicate that the main hurdle to using AAV vectors for *in vivo* gene delivery is the development of host immune responses to AAV proteins and/or the transgene product. These responses can destroy transgene-expressing cells and can limit the efficacy of vector readministration to increase transgene expression. Mice are relatively tolerant of AAV vector-mediated transduction and can express many vector-encoded foreign proteins for long periods. In contrast, robust immune responses to transgene products and to AAV capsid proteins that dramatically limit transgene expression have been demonstrated in dogs (see Halbert, et al., *Mol. Ther.* 18:1165-1172 (2010); Wang, et al., *Hum. Gene Ther.* 18:18-26 (2007); Wang, et al., *Mol. Ther.* 15:1160-1166 (2007), predicting that cellular immunity to AAV vectors will likely be an obstacle in humans. In line with the results observed in murine and canine studies, clinical trials in human subjects have identified problems that are often not observed in small animal models. For example, transient transaminitis developed shortly after vector administration in a trial of hepatic AAV2-mediated factor IX transfer in hemophilia B patients and was accompanied by the rise of AAV2 capsid-specific CD8⁺ T cells. In this trial, transgene expression was sustained for less than 8 weeks. Manno, C.S. et al., *Nat. Med.* 12:342-347 (2006).

In addition, several immunogenic epitopes of the capsid proteins of AAV2 and AAV8 have been identified in humans (Chen, et al., *Mol. Ther.* 13:260-269 (2006);

Manno et al., 2006)). These data indicate that additional studies of immunogenicity of AAV vectors are warranted so that strategies can be developed to permit long-term transgene expression.

The main protein components of AAV vector virions are the capsid proteins.

5 Immune responses against capsid proteins can arise in response to the large amount of AAV vector virions required for effective gene therapy. In addition, although AAV vectors are designed to be replication-defective and are generally assumed to be incapable of production of viral proteins, as demonstrated in Examples 1-3, the standard cap gene expression constructs used to make AAV vectors can be packaged and

10 expressed at low levels in cells exposed to AAV vectors, and could result in long-term stimulation of immune responses against capsid proteins in gene therapy recipients. As further described in Example 3, we have developed a method to prevent cap gene transfer and expression through the use of a Captron construct comprising a Cap expression cassette comprising at least one large intron. In this Example, a study was carried out in a

15 dog model to determine whether this Captron vector alteration results in reduced immune responses concomitant with a reduced need for immunosuppression.

Dogs are the only species commonly used in preclinical studies that has the rare combination of great morphologic diversity and a well-mixed gene pool, as do humans. Further, the canine model has an unexcelled track record of >40 years of translating

20 laboratory work in hematopoietic cell transplantation to the treatment of human patients with malignant and non-malignant blood disorders. The existence of a canine model for DMD, the *cxmd* dog, provided a model to test directly the efficacy of dystrophin replacement strategies to treat DMD. This Example describes an extended study designed to determine if AAV vectors generated using the captron based packaging system reduces

25 immune responses to AAV vectors designed to treat muscular dystrophy in *cxmd* dogs as a prelude to human clinical trials.

Methods:

For this study we used an AAV vector encoding canine factor IX (cFIX) driven by a human cytomegalovirus immediate-early promoter. We chose cFIX because this

30 protein can be readily detected in canine muscle, where it is not normally made, and cFIX is not immunogenic in dogs. The cFIX vector was produced in AAV6 capsids by using a standard cap6 expression plasmid (cap6) or one in which a large intron was inserted into the cap6 gene (captron plasmid), as described in Example 3, and were purified on heparin

columns, as described in Halbert and Miller, *Methods Mol. Biol.* 246:201-212 (2004), hereby incorporated herein by reference, or by centrifugation in CsCl. AAV6-CMV-cFIX vector titer was determined by quantitating vector genomes (VG). 10^{11} or 5×10^{11} VG in 250 μ l saline was injected per site into the hind-limb biceps muscles of canines underneath non-absorbable sutures to allow accurate biopsy. Canine experiments were performed as described in Wang et al., *Mol. Ther.* 15:1160-1166 (2007), hereby incorporated herein by reference. Biopsies were performed 4 weeks later. Serial sections were examined for analysis of muscle tissue structure and for detection of local mononuclear cell infiltration (i.e., T cell infiltration) using hematoxylin and eosin (H&E) staining and for cFIX expression and the presence of CD8 T-cells by immunohistochemistry.

Results:

Immunohistochemistry and H&E analysis was carried out on muscle biopsy samples obtained four weeks after administration of various preparations of AAV6-CMV-cFIX purified over a heparin column (results summarized in TABLE 8 below), or purified over a CsCl gradient (results summarized in TABLE 9 below).

TABLE 8: Summary of 4 week biopsy histology results for AAV6-CMV-cFIX preparations purified over a heparin Col.

	1 x 10^{11} VG generated using standard Cap6 packaging plasmid	5 x 10^{11} VG generated using Cap6 + intron (Captron) packaging plasmid	5 x 10^{11} VG generated using Cap6 + intron (Captron) packaging plasmid
cFIX (green stain)	+	+	+
H&E (evidence of inflammatory response)	++	-	-
CD8 (green stain)	++	-	-

TABLE 9: Summary of 4 week biopsy histology results for AAV6-CMV-cFIX preparations purified by centrifugation in CsCl

	1 x 10^{11} VG generated using standard Cap6	5 x 10^{11} VG generated using Cap6 + intron (Captron) packaging
--	--	--

	packaging plasmid	plasmid
cFIX (red or green stain)	+	+
H&E (evidence of inflammatory response)	++	-
CD8 (green stain)	++	-

TABLE 8 summarizes the results of immunohistochemistry and H&E analysis of four week biopsy samples after administration to canines of various preparations of AAV6-CMV-cFIX purified over a heparin column. As summarized in TABLE 8 (row 1), approximately equivalent levels of cells (green staining) were found to be expressing cFIX in muscle biopsy samples after transduction with an AAV cFIX vector preparation made using the standard cap6 plasmid as compared to the level of cFIX staining with an AAV cFIX vector preparation made using the captron plasmid. As shown in row 2, a relatively high level of inflammation in muscle biopsy samples was observed after transduction with the AAV cFIX vector preparation made using the standard cap6 plasmid, as determined by H&E staining, in comparison to the nearly complete muscle preservation in biopsy samples and lack of inflammation observed after transduction with the AAV cFIX vector preparation made using the captron plasmid. As shown in row 3, a higher level of CD8 cells (green staining) was observed in the biopsy sample after transduction with an AAV cFIX vector preparation made using the standard cap6 plasmid as compared to the very low level of CD8 cells present after transduction with an AAV cFIX vector preparation made using the captron plasmid.

TABLE 9 summarizes the results of immunohistochemistry and H&E analysis of four week biopsy samples after administration to canines of various preparations of AAV6-CMV-cFIX purified over a CsCl gradient. As summarized in TABLE 9 (row 1), approximately equivalent levels of cells (green staining) were found to be expressing cFIX in muscle biopsy samples after transduction with an AAV cFIX vector preparation made using the standard cap6 plasmid as compared to the level of cFIX staining with an AAV cFIX vector preparation made using the captron plasmid. As shown in row 2, a relatively high level of inflammation in muscle biopsy samples was observed after transduction with the AAV cFIX vector preparation made using the standard cap6 plasmid, as determined by H&E staining, in comparison to the nearly complete muscle preservation in biopsy samples and lack of inflammation observed after transduction with

the AAV cFIX vector preparation made using the captron plasmid. As shown in row 3, a higher level of CD8 cells (green staining) was observed in the biopsy sample after transduction with an AAV cFIX vector preparation made using the standard cap6 plasmid as compared to the very low level of CD8 cells present after transduction with an AAV cFIX vector preparation made using the captron plasmid.

These results indicate that a relatively high level immune response occurred in the dogs injected with the AAV cFIX vector prepared using the standard cap6 plasmid and dramatically reduced levels of immune response in the dogs injected with the AAV cFIX vector prepared using the captron plasmid. It is clear that cells transduced with the AAV cFIX vector generated using the captron plasmid express cFIX. It also appears that vector purification by CsCl centrifugation did not reduce the immune response observed in dogs injected with the AAV cFIX vector generated using the standard cap6 plasmid, as compared to the immune response observed with the AAV cFIX vector generated using the standard cap6 plasmid and heparin purification. This is an important result because vector production using heparin columns is more easily scaled up to the quantities required for clinical trials.

Overall Conclusion:

The results described in Examples 1-4 demonstrate that AAV vectors generated using the captron construct, which eliminates capsid gene transfer and capsid expression in transduced cells, are dramatically less immunogenic when administered to a dog at therapeutic *in vivo* amounts than AAV vectors generated using the standard cap6 construct. In view of these results, it is expected that AAV vectors made using captron plasmids will generate an increase in the number and persistence of cFIX (or other transgenes of interest) positive cells. It is also expected that AAV-captron generated vectors will reduce the level of, or eliminate the need for an immunosuppressive regimen after AAV vector transduction.

While illustrative embodiments have been illustrated and described, it will be appreciated that various changes can be made therein without departing from the scope of the invention.

CLAIMS

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. An intron-modified cap expression cassette useful for generating adeno-associated virus (AAV) vector particles, said expression cassette comprising:

(a) a nucleic acid molecule comprising a nucleotide sequence encoding an AAV capsid protein operably linked to a promoter, wherein the encoded AAV capsid protein is capable of packaging AAV vectors in the presence of rep and adeno helper functions, and

(b) at least one heterologous intron sequence operably linked to the nucleotide sequence encoding the AAV capsid protein;

wherein the total length of the expression cassette is at least 5 kilobases.

2. The expression cassette of Claim 1, wherein the nucleotide sequence encodes an AAV capsid protein selected from the group consisting of AAV serotypes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13, or a variant or hybrid protein thereof.

3. The expression cassette of Claim 1, wherein the nucleotide sequence encodes an AAV capsid protein from AAV-2 or AAV-6, or a variant or hybrid protein thereof.

4. The expression cassette of Claim 1, wherein the promoter is a heterologous promoter.

5. The expression cassette of Claim 1, wherein the total size of the at least one heterologous intron sequence(s) has a length of at least 2 kb.

6. The expression cassette of Claim 1, wherein the heterologous intron sequence is positioned between the promoter and the Cap open reading frame.

7. The expression cassette of Claim 1, wherein the heterologous intron sequence comprises at least one splice donor and at least one splice acceptor site.

8. The expression cassette of Claim 1, further comprising an AAV intron sequence adjacent to the 3' end of the heterologous intron sequence.

9. The expression cassette of Claim 8, wherein the heterologous intron sequence comprises at least one splice donor site and the AAV intron sequence comprises at least one splice acceptor site.

10. A vector comprising the expression cassette of Claim 1.

11. A cell comprising the expression cassette of Claim 1.

12. A method of generating an expression cassette for producing adeno-associated virus (AAV) vectors, comprising inserting at least one heterologous intron sequence into a nucleic acid molecule comprising a nucleotide sequence encoding an AAV capsid protein operably linked to a promoter, wherein the total length of the expression cassette is at least 5 kilobases.

13. The method of Claim 12, wherein the heterologous intron sequence has a length of at least 3 kb.

14. The method of Claim 12, wherein the heterologous intron sequence is positioned between the promoter and the Cap open reading frame.

15. A method of reducing cap gene contamination in a population of AAV vector particles, comprising:

- (a) introducing an AAV vector into a suitable host cell comprising the intron-modified cap expression cassette of Claim 1;
- (b) expressing Rep and adeno helper functions in the host cell; and
- (c) culturing the host cell to produce a population of AAV vector particles.

16. An AAV vector particle produced by the method of Claim 15.

17. The method of Claim 15, wherein the amount of DNA encoding Cap packaged in the AAV vector particles of step (c) is less than the amount of DNA encoding Cap packaged in a control AAV vector preparation prepared with an expression vector encoding Cap that does not contain a heterologous intron sequence.

18. The method of Claim 15, further comprising purifying at least a portion of the population of AAV vector particles produced in accordance with step (c) over a heparin column.

19. The method of Claim 15, wherein the AAV vector comprises a nucleic acid sequence encoding a therapeutic molecule under the control of sequences which direct expression thereof in a cell.

20. A method of reducing the immune response in a mammalian subject undergoing treatment with an AAV vector comprising:

administering to the mammalian subject a preparation of AAV vector particles comprising a nucleic acid sequence encoding a therapeutic molecule, wherein the preparation of AAV vector particles was produced with the intron-modified cap expression cassette of Claim 1, wherein the immune response in the mammalian subject to the AAV vector generated using the intron-modified cap expression cassette is significantly reduced or absent as compared to the immune response generated with a preparation of AAV vector particles generated using a standard cap expression cassette.

21. A complementation assay for measuring the presence or amount of cap gene contamination in a preparation of recombinant AAV vector virions comprising:

(a) infecting a host cell with a test preparation of AAV vector, wherein the host cell comprises:

- (i) an indicator AAV vector with a detectable marker,
- (ii) a rep expression vector; and
- (iii) adenovirus helper functions;

(b) culturing the host cell for a period of time sufficient to generate AAV vector virions from the indicator AAV vector;

(c) contacting an indicator cell with cell lysate obtained from the host cells cultured in accordance with step (b); and

(d) determining the presence or amount of indicator cells that are positive for the detectable marker, wherein a positive result indicates that the test preparation of AAV vector virions contains cap gene contamination.

22. A complementation assay for measuring the presence or amount of rep gene contamination in a preparation of AAV vector virions comprising:

(a) infecting a host cell with a test preparation of AAV vector, wherein the host cell comprises:

- (i) an indicator AAV vector with a detectable marker,
- (ii) a cap expression vector; and
- (iii) adenovirus helper functions;

(b) culturing the host cell for a period of time sufficient to generate AAV vector virions from the indicator AAV vector;

(c) contacting an indicator cell with cell lysate obtained from the host cells cultured in accordance with step (b); and

(d) determining the presence or amount of indicator cells that are positive for the detectable marker, wherein a positive result indicates that the test preparation of AAV vector virions contains rep gene contamination.

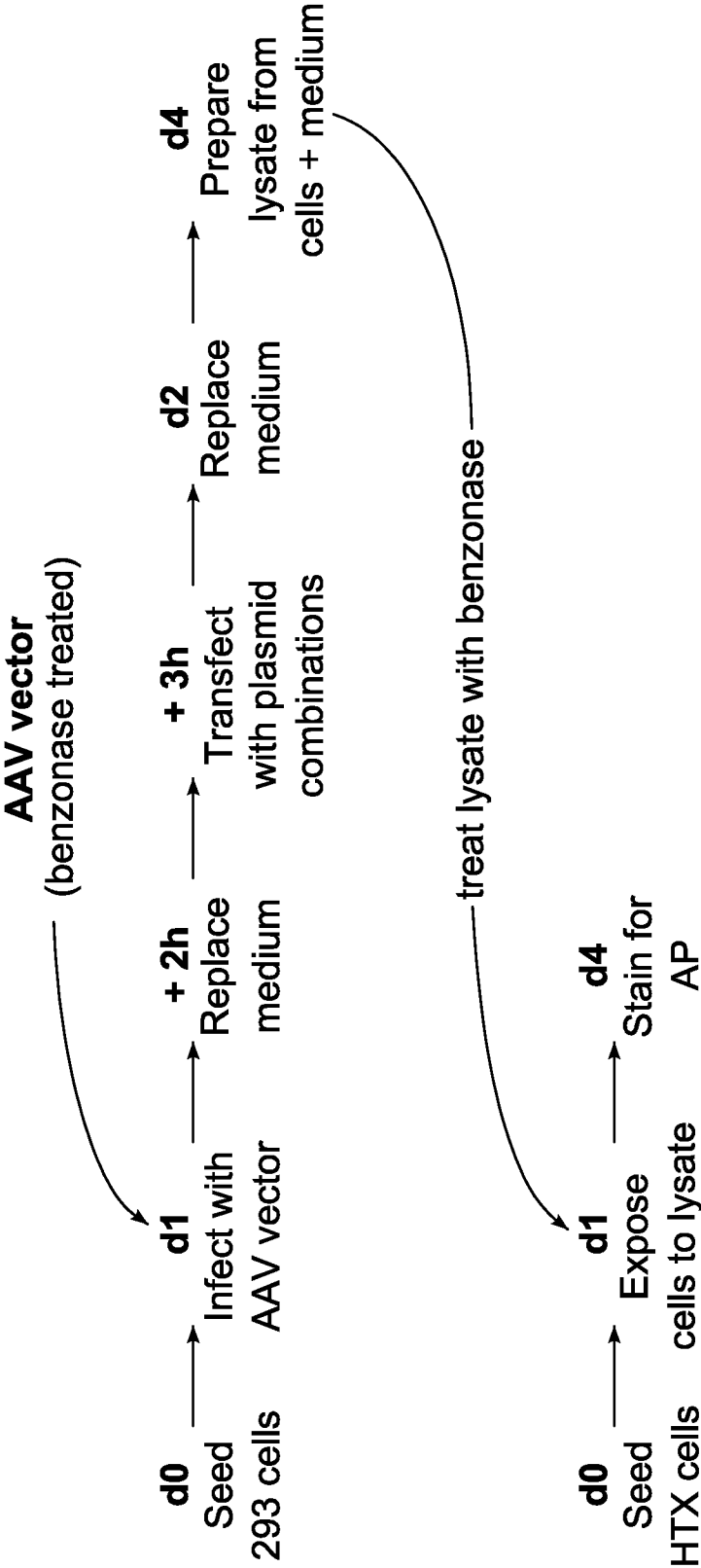


Fig. 1.

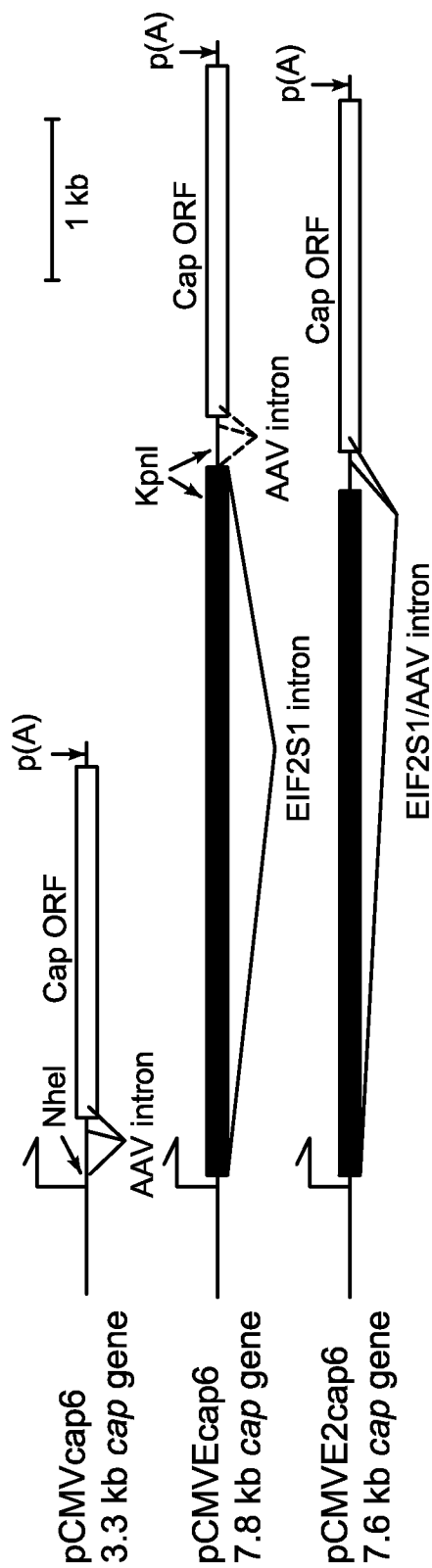


Fig. 2A.

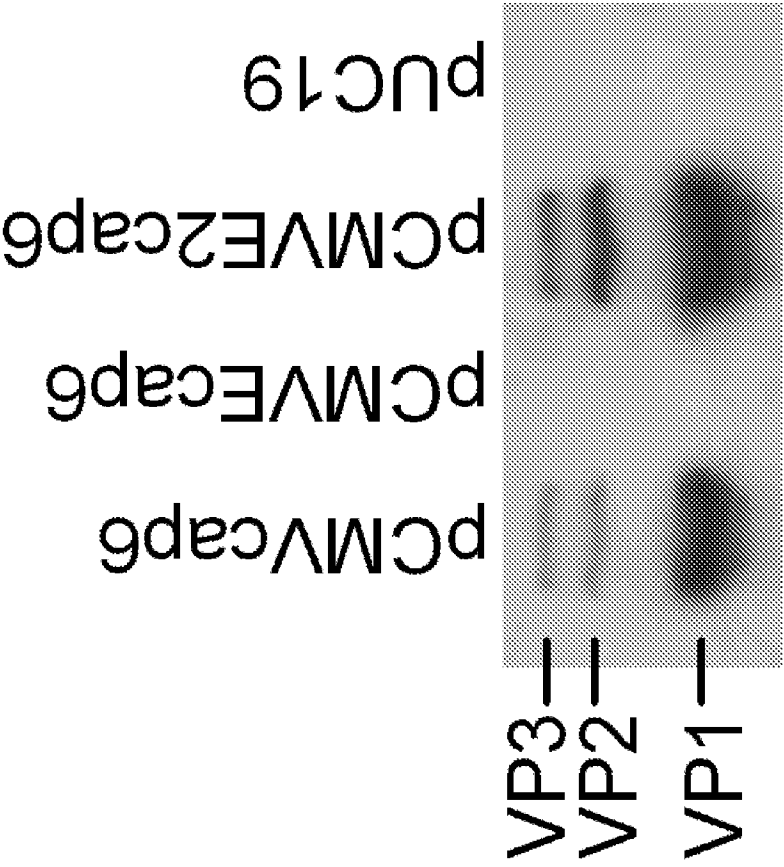


Fig. 2B.

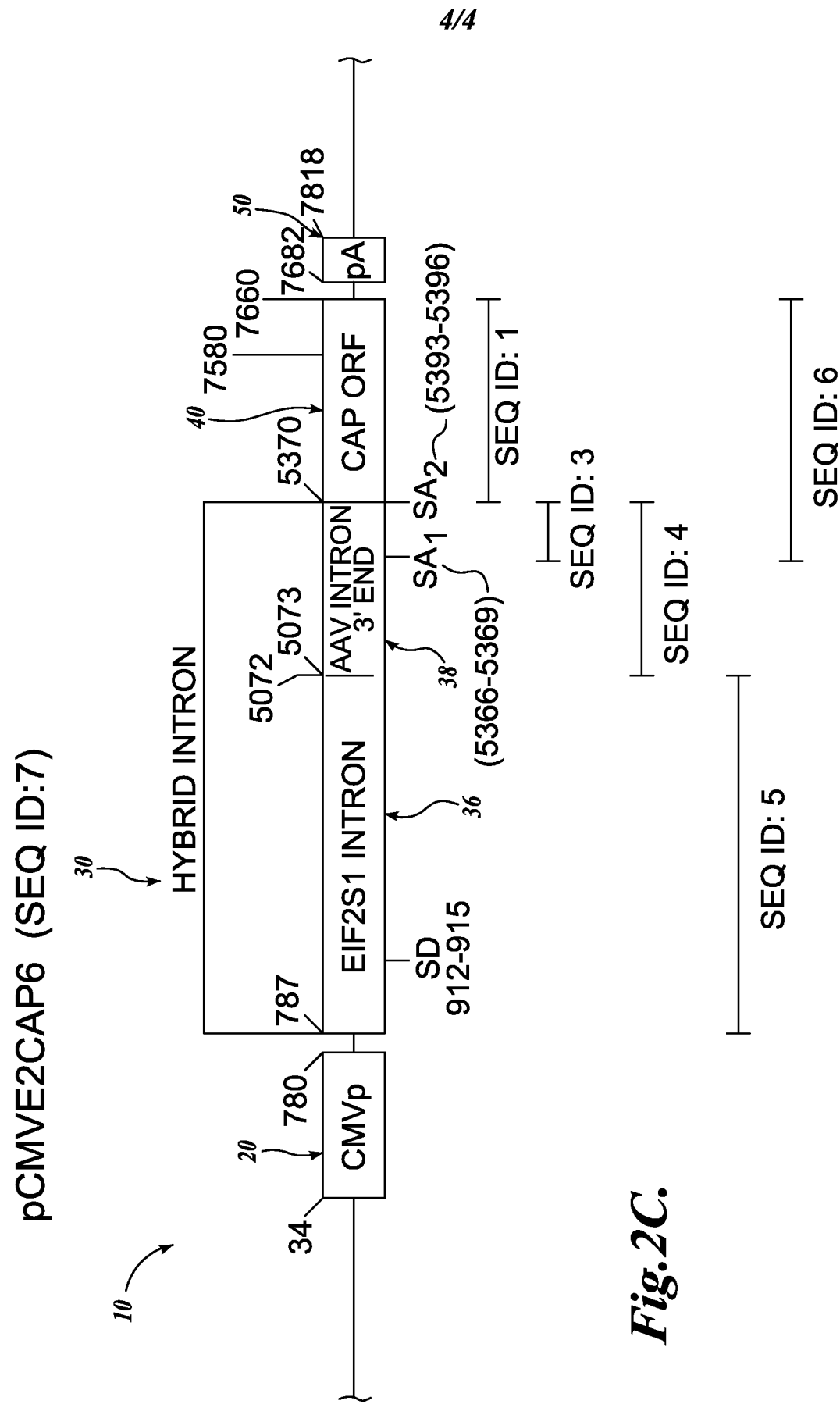


Fig. 2C.