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(54) **Title**: MONOMERIC DUPLEX AAV VECTORS

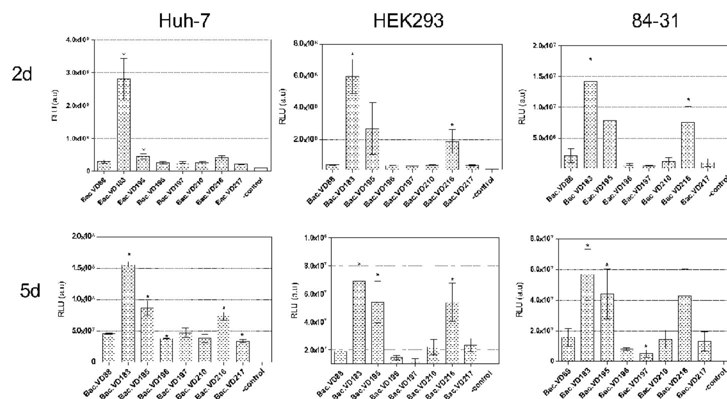

Fig. 4

(57) **Abstract**: The present invention relates to use of an insect cell capable of expressing AAV Rep proteins and AAV Cap proteins in the presence of a vector genome comprising at least one AAV ITR, wherein Rep52 and/or Rep40 protein expressionand/or activityis increased relative to Rep78 and/or Rep68 protein expressionand/or activity in the preparation of monomeric duplex AAV vector. The present invention relates to use of an insect cell capable of expressing AAV Rep proteins and AAV Cap proteins in the presence of a vector genome comprising at least one AAV ITR, wherein the nicking activity of Rep78 and/or Rep68 is reduced relative to the helicase/encapsidation activity of Rep52 and/or Rep40 in the preparation of monomeric duplex AAV vector. The invention further relates to corresponding methods for the preparation of monomeric duplex AAV vector.

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Monomeric duplex AAV vectors

Field of the invention

5 The invention also relates to the use of insect cells and Rep protein encoding sequences in the preparation of AAV vectors. The present invention relates to a method for the preparation of such AAV vectors. The invention relates to the AAV vectors themselves and to compositions, such as pharmaceutical compositions comprising the AAV vectors. The invention further relates to methods in which such 10 AAV vectors and compositions are used and to uses of the AAV vectors and compositions.

Background of the invention

15 In recent years, adeno-associated virus (AAV) vector production by means of baculovirus-based expression systems (BEVS) in insect cells has become increasingly popular since the system is readily scalable for industrial applications in gene therapy.

In this production system, recombinant baculoviruses are used encoding the AAV rep genes, the AAV cap genes and a vector genome flanked by AAV ITRs. 20 Infection of suspension culture of insect cells with a mixture of the three recombinant baculoviruses allows the entry of a combination of the essential AAV viral sequences, vector genome and helper functions in the producer cells. Recombinant AAV (rAAV) particles may then be packaged and assembled. The rAAV particles contain a vector genome which typically comprises one or more transgenes, flanked at both ends by the 25 ITR regions, in the form of single-stranded DNA. Replication of recombinant baculovirus in the host insect cells, generally ends in the lysis and death of the infected cells after a few days. The resulting viruses (baculovirus and rAAV particles) are either in part released into the cell culture supernatant or else remain in the lysed cells.

The requirement for complementary-strand synthesis, or recruitment, is 30 considered to be a significant limiting factor in the efficiency of rAAV vectors. Expression from rAAV in liver tissue coincides with the formation of duplex DNA and the virion DNA appears to be lost if not converted to duplex within 5-13 weeks.

Accordingly, the present invention addresses a need in the art for improved parvovirus gene delivery vectors. In particular the present invention addresses the requirement for complementary strand synthesis by conventional AAV gene delivery vectors.

Brief description of the invention

Adeno-associated virus (AAV) is a nonpathogenic, helper dependent member of the parvovirus family. One of the identifying characteristics of this group is the encapsidation of a single-stranded DNA (ssDNA) genome. In the case of AAV, the separate plus or minus polarity strands are packaged with equal frequency, and either is infectious. At each end of the ssDNA genome, a palindromic terminal repeat (ITR) structure base-pairs upon itself into a hairpin configuration. This serves as a primer for cellular DNA polymerase to synthesize the complementary strand after uncoating in the host cell.

AAV is a very attractive candidate for use in the preparation of virus-based gene therapy vectors. In order that it can be used in such applications, the vector should be easily available in high quantity and should be as potent as possible. However, the requirement for complementary-strand synthesis, or recruitment, is considered to be a significant limiting factor in the efficiency of rAAV vectors. The invention addresses this draw-back by providing a method for the preparation of monomeric duplex containing AAV vectors. Such vectors comprise a double stranded vector genome and are thus not limited by the necessity for complementary-strand synthesis to take place.

According to the invention, these is thus provided use of an insect cell capable of expressing AAV Rep proteins and AAV Cap proteins in the presence of a vector genome comprising at least one AAV ITR, wherein Rep52 and/or Rep40 protein expression and/or activity is increased relative to Rep78 and/or Rep68 protein expression and/or activity in the preparation of monomeric duplex AAV vector.

The invention also provides use of an insect cell capable of expressing AAV Rep proteins and AAV Cap proteins in the presence of a vector genome comprising at least one AAV ITR, wherein the nicking activity of Rep78 and/or Rep68 is reduced relative to the helicase/encapsidation activity of Rep52 and/or Rep40 in the preparation of monomeric duplex AAV vector.

In addition, the invention provides a method for the preparation of monomeric duplex AAV vector, said method comprising expressing in insect cells AAV Rep proteins and AAV Cap proteins in the presence of a vector genome comprising at least 5 one AAV ITR, wherein Rep52 and/or Rep40 protein expression and/or activity is increased relative to Rep78 and/or Rep68 protein expression and/or activity.

The invention further provides a method for the preparation of monomeric duplex AAV vector, said method comprising expressing in insect cells AAV Rep proteins and AAV Cap proteins in the presence of a vector genome construct flanked by at least one 10 AAV ITR, wherein the nicking activity of Rep78 and/or Rep 60 is reduced relative to the helicase/encapsidation activity of Rep52 and/or Rep 40.

The invention further provides:

- a method for the preparation of monomeric duplex AAV vector, said method comprising expressing in insect cells AAV Rep proteins and AAV Cap proteins in the 15 presence of a vector genome comprising at least one AAV ITR, wherein said Rep proteins are expressed from a sequence as set out in any one of SEQ ID NOs: 1 to 7;
- a method for the preparation of monomeric duplex AAV vector, said method comprising expressing in insect cells AAV Rep proteins and AAV Cap proteins in the presence of a vector genome comprising at least one AAV ITR, wherein said Rep 20 proteins are expressed from a construct as set out in Fig. 1 as Bac.VD183;
- use of an insect cell capable of expressing AAV Rep proteins and AAV Cap proteins in the presence of a vector genome comprising at least one AAV ITR, wherein the Rep proteins are expressed from a nucleotide sequence as set out in any one of SEQ ID NOs: 1 to 7 in the preparation of monomeric duplex AAV vector;
25 - use of an insect cell capable of expressing AAV Rep proteins and AAV Cap proteins in the presence of a vector genome comprising at least one AAV ITR, wherein the Rep proteins are expressed from a construct as set out in Fig. 1 as Bac.VD183 in the preparation of monomeric duplex AAV vector;
- use of a Rep encoding sequence according to any one of SEQ ID NOs: 1 to 7 in 30 the preparation of monomeric duplex AAV vector in insect cells;
- use of a construct as set out in Fig. 1 as Bac.VD183 in the preparation of monomeric duplex AAV vector in insect cells;
- a monomeric duplex AAV vector produced by a method or use of the invention;

- a composition comprising AAV vector of which at least about 70% is in the form of monomeric duplex AAV vector;
- a pharmaceutical composition comprising monomeric duplex or a composition of the invention and a pharmaceutically acceptable carrier or diluent;
- a method for delivering a nucleotide sequence to a cell, which method comprises contacting monomeric duplex, a composition or a pharmaceutical composition of the invention under conditions such that the monomeric duplex AAV enters the cell;
- a method for administering a nucleotide sequence to a subject, which method comprises adminstering to the said subject monomeric duplex, a composition or a pharmaceutical composition of the invention; and
- use of monomeric duplex, a composition or a pharmaceutical composition of the invention in the manufacture of a medicament for use in the administration of a nucleotide to a subject.

Brief description of the drawings

Figure 1 shows the organisation of the Bac.rep baculovirus constructs: Bac.VD88 expresses rep78/52 by a Polyhedron promoter. Rep52 is expressed from the internal rep78 sequence. Bac.VD183 is derived from pFBDSLR (Urabe et al., 2002) however, has a codon optimized rep52 sequence. Bac.VD195, Bac.VD196 and Bac.VD197 are derivatives from Bac.VD88 and differ either from initiation codon (ATG or ACG for rep78 expression) or ATG codon mutations in the unique rep78 sequence. Bac.VD210, Bac.VD216 or Bac.VD217 are derived from Bac.VD88 by error prone PCR techniques. These rep mutants are described in co-pending US provisional patent application no. 61/312845.

Figure 2 shows Rep78/52 expression of the various Bac.rep constructs is shown on Western blot on day 1 and day 2 following start of vector production. A low expression of rep78 and high expression of rep52 on the first and second day of infection is only seen for Bac.VD183 and Bac.VD197. All other Baculovirus rep-expressing constructs show higher rep78 levels compared to rep52 levels.

Figure 3 shows vector DNA containing a transgene-expression cassette of 3.5 kb is isolated from purified AAV vector preparations made by the use of the various rep-baculoviruses. The DNA is monitored following alkaline gel electrophoresis.

Monomeric duplex (i.e double stranded vector DNA and monomeric DNA, i.e. single stranded vector DNA, although on the alkaline gel everything is single stranded) is detected on gel. For Bac.VD183 shows the highest enrichment in monomeric duplex vector DNA as compared to monomer DNA.

5 Figure 4 shows three cell lines which were infected with AAV vector preparation containing a CMV-SEAP expression cassette and made using the various Bac.rep constructs. The activity of SEAP produced in the cell lines at 2 and 5 days after infection was measured. The vector preparation made with the Bac.VD183 baculovirus shows the highest transduction efficiency in all cell lines.

10 Figure 5 shows Western blot detection of rep78/52 proteins during vector production of rAAV-Luc or rAAV-Reporter gene 2 using Bac.VD183 or Bac.VD216 in different ratio to Bac.cap and Bac.vector (i.e. 1:1:1, 2:1:1, 3:1:1 or 4:1:1). It should be noted that rep78 expression is low compared to rep52 when Bac.VD183 is used whereas the kinetics are reversed for Bac.VD216 (i.e rep78 is high compared to rep52 15 on day 2) after infection.

Figure 6 shows Southern blot of AAV vector preparations made using FBDSLR (Urabe et al., 2002) (A), Bac.VD216 (B) or Bac.VD183 (C). Only monomeric vectors are detectable in A, similar amount of monomeric duplex and monomeric vector genomes are detected in B, whereas high amount of monomeric duplex vector genomes 20 are detected in C.

Figure 7 shows transduction of AAV-Luciferase vector on Huh7 cells at 2 and 7 days post-infection. rAAV-Luciferase vector was produced using Bac.183, Bac.216, FBDSLR in increasing amount compared to Bac.Cap and Bac.Luciferase. Vector prepared with Bac.183 shows the highest potency as compared to two other vector 25 preparations. Optimal transduction efficiency is obtained when Bac.VD183 used for generation of rAAV is added to the cell culture in a three fold vector access to Bac.cap and Bac.Luc.

Figure 8 shows an alkaline agarose gel of vector DNA derived from vector preparation using Bac.VD183 or Bac.VD88, a baculovirus containing a vector 30 construct of a size of 1 kb (mU1) and a Bac.Cap6 (serotype 6) . Because of the small vector genome size (1 kb) more than two copies of the transgene DNA can be packaged into the capsid only when Bac.VD183 is used.

Brief description of the sequence listing

SEQ ID NO: 1 sets out the sequence of the VD88 variant of AAV2 Rep protein encoding sequence.

5 SEQ ID NO: 2 sets out the sequence of the VD195 variant of AAV2 Rep protein encoding sequence.

SEQ ID NO: 3 sets out the sequence of the VD196 variant of AAV2 Rep protein encoding sequence.

SEQ ID NO: 4 sets out the sequence of the VD197 variant of AAV2 Rep protein 10 encoding sequence.

SEQ ID NO: 5 sets out the sequence of the VD210 variant of AAV2 Rep protein encoding sequence.

SEQ ID NO: 6 sets out the sequence of the VD216 variant of AAV2 Rep protein encoding sequence.

15 SEQ ID NO: 7 sets out the sequence of the VD217 variant of AAV2 Rep protein encoding sequence.

SEQ ID NO: 8 sets out the sequence of the Rep58 variant used in the VD183 construct.

SEQ ID NO: 9 sets out the sequence of the Rep78 variant used in the VD183 20 construct.

SEQ ID NO: 10 sets out the full length nucleotide sequence of Rep78 from AAV2.

SEQ ID NO: 11 sets out the full length amino acid sequence of Rep78 from AAV2.

25

Detailed description of the invention

The present invention relates to reagents for gene delivery, to methods for their preparation and uses of certain regents in their preparation. More particularly, the 30 present invention relates to the use of insect cells and Rep protein encoding sequences for the preparation of parvovirus-based gene delivery vectors and to an improved method for the preparation of parvovirus-based gene delivery vectors. In particular, the parvovirus-based gene delivery vector may be an adeno-associated virus (AAV).

As used herein, the term "vector" or "gene delivery vector" may refer to a parvovirus (e. g., AAV) particle that functions as a gene delivery vehicle, and which comprises DNA (i. e., the vector genome) packaged within a parvovirus (e.g., AAV) capsid. Alternatively, in some contexts, the term "vector" may be used to refer to the 5 vector genome/virion DNA.

As used herein, a "parvovirus vector genome" or "recombinant parvovirus vector genome" is typically a parvovirus genome (i.e., vDNA) into which a heterologous (e. g., foreign) nucleotide sequence (e. g., transgene) has been inserted. A "recombinant parvovirus particle" comprises a recombinant parvovirus vector genome packaged 10 within a parvovirus capsid.

Likewise, a "rAAV vector genome" is an AAV genome (i. e., virion DNA) that comprises a heterologous nucleotide sequence. rAAV vectors require only the 145 base terminal repeats in *cis* to generate virus. All other viral sequences are dispensable and may be supplied in *trans*. Typically, the rAAV vector genome will only retain the 15 minimal terminal repeat (ITR) sequences so as to maximize the size of the transgene that can be efficiently packaged by the vector. A "rAAV particle" comprises a rAAV vector genome packaged within an AAV capsid.

Typically, the vector genome comprises a heterologous nucleotide sequence(s) (as described below) to be packaged for delivery to a target cell. According to this 20 particular embodiment, the heterologous nucleotide sequence is located between the viral ITRs at either end of the substrate. In further preferred embodiments, the parvovirus (e. g., AAV) cap genes and parvovirus (e.g., AAV) rep genes are deleted from the template (and the vDNA produced therefrom). This configuration maximizes the size of the heterologous nucleic acid sequence (s) that can be carried by the 25 parvovirus capsid.

Accordingly, the present invention relates to methods for the preparation and of animal parvoviruses, in particular dependoviruses such as infectious human or simian AAV, and the components thereof (e.g., an animal parvovirus genome) for use as vectors for introduction and/or expression of nucleic acids in mammalian cells. In 30 particular, the invention relates to improvements in productivity of such parvoviral vectors, especially AAV, when produced in insect cells.

Viruses of the Parvoviridae family are small DNA animal viruses. The family Parvoviridae may be divided between two subfamilies: the Parvovirinae, which infect

vertebrates, and the Densovirinae, which infect insects. Members of the subfamily Parvovirinae are herein referred to as the parvoviruses and include the genus Dependovirus. As may be deduced from the name of their genus, members of the Dependovirus are unique in that they usually require coinfection with a helper virus
5 such as adenovirus or herpes virus for productive infection in cell culture. The genus Dependovirus includes AAV, which normally infects humans (e.g., serotypes 1, 2, 3A, 3B, 4, 5, and 6) or primates (e.g., serotypes 1 and 4), and related viruses that infect other warm-blooded animals (e.g., bovine, canine, equine, and ovine adeno-associated viruses). Further information on parvoviruses and other members of the Parvoviridae is
10 described in Kenneth I. Berns, "Parvoviridae: The Viruses and Their Replication," Chapter 69 in Fields Virology (3d Ed. 1996). For convenience the present invention is further exemplified and described herein by reference to AAV. It is however understood that the invention is not limited to AAV but may equally be applied to other parvoviruses.

15 The genomic organization of all known AAV serotypes is very similar. The genome of AAV is a linear, single-stranded DNA molecule that is less than about 5,000 nucleotides (nt) in length. Inverted terminal repeats (ITRs) flank the unique coding nucleotide sequences for the non-structural replication (Rep) proteins and the structural (VP) proteins. The VP proteins (VP1, -2 and -3) form the capsid. The terminal 145 nt
20 are self-complementary and are organized so that an energetically stable intramolecular duplex forming a T-shaped hairpin may be formed. These hairpin structures function as an origin for viral DNA replication, serving as primers for the cellular DNA polymerase complex. Following wtAAV infection in mammalian cells the Rep genes (i.e. Rep78 and Rep52) are expressed from the P5 promoter and the P19 promotor,
25 respectively and both Rep proteins have a function in the replication of the viral genome. A splicing event in the Rep ORF results in the expression of actually four Rep proteins (i.e. Rep78, Rep68, Rep52 and Rep40). However, it has been shown that the unspliced mRNA, encoding Rep78 and Rep52 proteins, in mammalian cells are sufficient for AAV vector production. Also in insect cells the Rep78 and Rep52
30 proteins suffice for AAV vector production.

A "recombinant parvoviral or AAV vector" (or "rAAV vector") herein refers to a vector comprising one or more polynucleotide sequences of interest, genes of interest or "transgenes" that are flanked by parvoviral or AAV inverted terminal repeat

sequences (ITRs). Such rAAV vectors can be replicated and packaged into infectious viral particles when present in an insect host cell that is expressing AAV rep and cap gene products (i.e. AAV Rep and Cap proteins). When an rAAV vector is incorporated into a larger nucleic acid construct (e.g. in a chromosome or in another vector such as a
5 plasmid or baculovirus used for cloning or transfection), then the rAAV vector is typically referred to as a "pro-vector" which can be "rescued" by replication and encapsidation in the presence of AAV packaging functions and necessary helper functions.

Recombinant adeno-associated (rAAV) vectors contain a linear single stranded
10 DNA genome which is flanked by inverted terminal repeats (ITRs). This DNA needs to be converted into double stranded form in order to be transcriptionally active in the host cell, which, as set out above, appears to be a rate limiting step leading to low transduction efficiency in some cell types. Recombinant AAV (rAAV) gene delivery vectors also package ssDNA of plus or minus polarity, and must rely on cellular
15 replication factors for synthesis of the complementary strand. While it was initially expected that this step would be carried out spontaneously, by cellular DNA replication or repair pathways, this does not appear to be the case. Early work with rAAV vectors revealed that the ability to score marker gene expression was dramatically enhanced when cells were co-infected with adenovirus, or transiently pretreated with genotoxic
20 agents. This enhancement correlated with the formation of duplex DNA from the single-stranded virion DNA (vDNA).

This critical step of double-stranded DNA formation may be bypassed through the use of self-complementary AAV vectors (see, for example, WO01/92551). The need for ssDNA to dsDNA conversion via either self paring of plus and minus strands
25 or DNA synthesis may be circumvented by packaging both complementary stands as one covalently linked molecule. This may be achieved by enhancing the production and encapsidation of dimeric genomes. Monomeric AAV genomes are produced upon the resolution of the two ITR’s at their terminal resolution sites (trs) by replicase enzyme. Upon deletion of one of the two terminal resolution sites at one of the ITRs,
30 Rep proteins are unable to generate the ssDNA nick at that site, which in turns increases generation of dimeric genomes. A dimeric genome will then contain a faulty ITR in the “middle” of the molecule and two flanking wild type ITRs, which will allow

for normal rounds of replication. Ultimately, this may result in the packaging of a dimer ("self-complementary AAV" or "scAAV") genome instead of a monomer.

Typically, a mutation of one ITR results in yields of 90% dimeric genomes. However, this technology allows for encapsidation of only two covalently linked copies of the genome.

The small transgene size used for some gene therapies, using RNAi or exon skipping technology, for example, gives rise to an opportunity to encapsidate multiple copies (eg. up to four copies when a vector genome of approximately 1kb is used) of covalently linked genomes in complementary form. This, however, cannot be performed by already existing technologies. The deletion of one trs site results in formation of maximally two covalently linked copies and thereby a new molecular design is needed. Moreover, the existing scAAV approach does not appear to be especially effective in insect cells, as compared to mammalian cells in which it was originally described.

Accordingly, the invention concerns use and a method in which multiple trs sequences are skipped and multimeric covalently linked genomes are encapsidated. The final size of the genome is then limited by the physical maximum capacity of the capsid and not by two flanking ITRs. This allows for the maximum encapsidation of four consecutive covalently linked copies of genome in the case of a transgene related to DMD treatment (~1kbp) for example.

We have now defined conditions that enable the production of vectors comprising monomeric duplex genomes. These vectors are referred to herein as monomeric duplex AAV vectors or monomeric duplex parvoviral AAV vectors. That is to say, such vectors are vectors which contain or comprise monomeric duplex genomes. These are the conditions that, during encapsidation of the vector genome, lead to skipping of the trs by Rep enzyme(s). This in turn results in encapsidation of a replicative form of the monomer, i.e. two covalently linked genomes. Furthermore, these conditions allow multiple trs sequences to be skipped such that multimeric covalently linked genomes may be encapsidated.

In the method of the invention, insect cells are used which, under the conditions defined in the method, allow skipping of trs resulting in lack of inverted terminal repeat resolution.

In the insect cell AAV production system, recombinant AAV (rAAV) is generated and encapsidated by two Rep proteins, large Rep78 and small Rep52. These two enzymes have multiple functions and can interact with each other. Rep78 is important in replication and trs resolution, whereas Rep52 plays a major role during encapsidation of replicated DNA. Rep52 has been shown to be not important for the replication reactions, whereas Rep78, although not indispensible, seems to be required for efficient encapsidation. It is known that during replication of the AAV genome, a variety of replication intermediates may be formed. These intermediate forms can consist of multiple covalently linked genomes (e.g. replicative form monomer containing two covalently linked genomes, replicative form dimer –four, etc.). The replicated DNA serves as a substrate for Rep complexes. It first needs to be unwound to a single stranded form and subsequently physically translocated through the pore into the capsid. During or after encapsidation of wild type AAV genome, Rep78 will nick at the terminal resolution site of replication intermediate allowing the encapsidation process to terminate. However, if the encapsidated genome size is half of the maximum encapsidation capacity of wild type AAV, the central ITR can be skipped by Rep78, resulting in the encapsidation of a 4.7kbp fragment containing two copies of the genome.

We have shown that the equilibrium between encapsidation and nicking can be influenced and shifted towards reduction of nicking activity of Rep78 during encapsidation process. This in turn results in the preferential packaging of multimeric genomes.

We have shown that encapsidation of multimeric genomes observed with various Rep proteins relates to the ratio between large and small Rep proteins used for rAAV generation. Moreover, the biphasic expression of replicase enzymes which is stronger for Rep78 at the first phase of production and for Rep52 at the second phase also contributes to the observed increase encapsidation of multimeric genomes.

The single-stranded nature of the AAV genome may impact the expression of rAAV vectors more than any other biological feature. Rather than rely on potentially variable cellular mechanisms to provide a complementary-strand for rAAV vectors, the method of the invention allows this problem to be circumvented by packaging both strands as a single DNA molecule.

All of these biological attributes support the generation and characterization of parvovirus vectors (delivering monomeric duplex DNA) that significantly contribute to the ongoing development of parvovirus-based gene delivery systems.

Thus, the use and method of the invention, allows packaging strands of plus and minus polarity tethered together in a single molecule. Accordingly, the present invention provides a parvovirus particle comprising a parvovirus capsid (e. g., an AAV capsid) and a vector genome, typically encoding a heterologous nucleotide sequence, where the vector genome is in the form of a monomeric duplex, i.e., the vector genome is in the form of a monomeric duplexed inverted repeat.

As will be evident to the skilled person, the term “monomeric duplex” covers any vector genome as packaged in a capsid which can form double-stranded DNA from a single molecule. The monomeric duplex may be capable of forming a wholly or partially double-stranded molecule. That is to say, a monomeric duplex is a vector genome in which one or more regions of heterologous sequence, preferably coding sequence, may form intra-strand base pairs. Generally, a monomeric vector genome of the invention contains a 5' and 3' terminus, (i.e., is not circular).

The region (s) of intra-strand complementarity (i.e., the region (s) which form intra-strand base pairs) in the monomeric duplex vector genome is typically positionally and/or quantitatively sufficient to enhance expression of a nucleotide sequence of interest contained within the vector as compared to a vector that is structurally analogous except for the position and/or quantity of base pairing, such that the analogous vector lacks sufficient intra-strand complementarity to enhance expression of the nucleotide sequence of interest.

Preferably, the region (s) of intra-strand base pairing are within the coding region (s), i.e., the intra-strand base pairing occurs within a nucleotide sequence that is to be expressed. The entire coding region(s) may be base paired.

The regions of intra-strand complementarity may be anywhere along the heterologous sequence and may be any of a number of sizes, in terms of contiguous nucleotides. Further, it is understood that the region or sequence of intra-strand base pairing may or may not be within a coding region of a heterolgous sequence. In some embodiments, a region(s) may be adjacent to, or, alternatively, near to, a (5'or 3') terminus. In other embodiments, a region (s) is adjacent to, or near to, the center of the monomeric duplex vector genome.

A region of intra-strand complementarity can be at least about any of the following: 5, 10, 15, 20, 25, 30, 35,40, 45,50,60, 75, 85,100, 110, 120,130, 145, 150, 160, 175, 200, 250, 300, 350, 400, 450, 500 or more contiguous nucleotides. The total amount of sequence that forms intra-strand base pairs (which could be in one or more regions) may be greater than about 125 nucleotides, greater than about 250 nucleotides, greater than about 500 nucleotides, and/or greater than about 1,000 nucleotides.

If a heterologous sequence contains more than one such region, the regions may be separated by several to several hundred nucleotides. Most preferably, the region should encompass the sequence for which expression (i. e., transcription and/or translation) is desired. For example, a monomeric duplex vector genome may contain two sequences for anti-sense expression, or two small genes, either contiguous (i. e., no intervening nucleotides) or separated by non-coding nucleotides. The monomeric duplex vector genome may also contains complementary sequences (in opposite orientation, to allow base pairing) for the two "coding" sequences.

A "heterologous nucleotide sequence" will typically be a sequence that is not naturally-occurring in the virus. Alternatively, a heterologous nucleotide sequence may refer to a viral sequence that is placed into a non-naturally occurring environment (e. g., by association with a promoter with which it is not naturally associated in the virus).

Accordingly, the invention provides a use and a method for the preparation of monomeric duplex containing AAV vector. That is to say, the use and method are ones which permits the preparation of monomeric duplex AAV vector, i.e. AAV vector comprising an AAV genome in the form of a monomeric duplex.

In general, the use and method of the invention allow the production of a recombinant parvoviral (rAAV) virion (comprising monmeric duplexed vector) in an insect cell. Preferably, the use and method comprise the steps of: (a) culturing an insect cell as defined in herein above under conditions such that recombinant parvoviral (rAAV) vector is produced; and, (b) recovery of the recombinant parvoviral (rAAV) vector. It is understood here that the recombinant parvoviral (rAAV) vector produced in the method preferably is an infectious parvoviral or AAV virion that comprise the recombinant parvoviral (rAAV) vector nucleic acids. Growing conditions for insect cells in culture, and production of heterologous products in insect cells in culture are well-known in the art and described e.g. in the above cited references on molecular engineering of insects cells.

Typically then, use and the method of the invention for producing a monomeric duplexed parvovirus particle, comprise: providing to a cell permissive for parvovirus replication (a) a nucleotide sequence encoding a template for producing vector genome of the invention (as described in detail herein); (b) nucleotide sequences sufficient for replication of the template to produce a vector genome; (c) nucleotide sequences sufficient to package the vector genome into a parvovirus capsid, under conditions sufficient for replication and packaging of the vector genome into the parvovirus capsid, whereby duplexed parvovirus particles comprising the vector genome encapsidated within the parvovirus capsid are produced in the cell. Preferably, the parvovirus replication and/or capsid coding sequences are AAV sequences.

In sum, the viral template to be replicated and packaged, parvovirus cap genes, appropriate parvovirus rep genes, and (preferably) helper functions are provided to a cell (e. g., a permissive or packaging cell) to produce parvovirus particles carrying the monomeric duplexed genome (i. e., the genome is capable of forming a"snap back"or self-complementary DNA after viral uncoating). The combined expression of the rep and cap genes encoded by the template and/or the packaging vector (s) and/or the stably transformed packaging cell results in the production of a parvovirus particle in which a parvovirus capsid packages a duplexed parvovirus genome according to the invention. The duplexed parvovirus particles are allowed to assemble within the cell, and may then be recovered by any method known by those of skill in the art.

The use and method involve expressing in insect cells AAV Rep proteins and AAV Cap proteins in the presence of a vector genome comprising at least one AAV ITR, wherein total Rep52 and Rep40 protein expression is increased relative to total Rep78 and Rep68 protein expression.

Wild type AAV express four Rep proteins as a result of splicing, two large Rep's: Rep78, Rep68 and two small Rep's: Rep52 and Rep40. It has been demonstrated that Rep78 and Rep52 are sufficient to generate rAAV. Accordingly, in the invention Rep78 and Rep52 may be used alone or in combination with one or both of Rep68 and Rep40. The total effect of the Rep proteins used is typically crucial. Rep68 and Rep 40 may be used alone or in combination with one or more of Rep78 and Rep52. Rep78 may be used together with Rep40 (in the absence of Rep68 and Rep52). Rep68 may be used together with Rep52 (in the absence of Rep78 and Rep40).

Also, Rep52 and Rep40 have slightly different enzymatic activities. Bothe enzymes are helicases and exhibit 3'-5' helicase activity. In addition Rep40 can perform unwinding of DNA in the 5'-3' direction and DNA which is blunt ended. One strategy to achieve generation of multiself complementary AAV is reduction of nicking activity during encapsidation of replicated transgene DNA. An alternative is to protect against nicking, which can be realized by increasing encapsidation of substrate DNA or it's unwinding. The single stranded DNA was shown to be much poorer substrate for Rep78 than the double stranded DNA. Thereby expression of Rep40 from strong late promoter e.g. polH could contribute to increase conversion of dsDNA into ssDNA, which as a result would be cleaved less efficient by Rep78.

Typically, total Rep52 and Rep40 protein expression is greater than total Rep78 and Rep68 expression.

Protein expression here is intended to cover greater enzymatic activity of Rep52/40 as compared with Rep78/68, for example on a molar basis, i.e. there may be a greater expression of Rep52/40 on a molar basis than Rep78/68. Protein expression of Rep52/40 may be about 1.5 times, about 2 times, about 4 times, about 5 times, about 10 times, about 20 times, about 50 times or more greater than that of the expression of Rep78/68.

The method of the invention may comprise expressing in insect cells AAV Rep proteins and AAV Cap proteins in the presence of a vector genome construct flanked by at least one AAV ITR (i.e. a vector genome comprising at least one AAV ITR), wherein the nicking activity of Rep78 and/or Rep68 is reduced relative to the helicase/encapsidation activity of Rep52 and/or Rep40.

Typically, the total nicking activity of Rep78 and/or Rep 68 is less than that of the total helicase/encapsidation activity of Rep52 and/or Rep 40.

Activity here is intended to cover greater enzymatic activity of Rep52/40 as compared with Rep78/68, for example on a molar basis or in terms of moles of substrate converted per unit time. Thus a greater amount of substrate may be converted (on a molar basis) by helicase/encapsidation activity in the cell by Rep52/40 than the amount of substrate converted (on a molar basis) by the nicking activity of Rep78/68.

Helicase/encapsidation activity of Rep52/40 may be about 1.5 times, about 2 times, about 4 times, about 5 times, about 10 times, about 20 times, about 50 times or more greater than that of the nicking activity of Rep78/68.

In the use and method of the invention, the vector genome is flanked by at least one ITR and, more typically, by two AAV ITRs (generally with one either side of the vector genome, i.e. one at the 5' end and one at the 3' end). There may be intervening sequences between any heterologous nucleotide sequence in the vector genome and one of more of the ITRs. That is to say a vector genome may comprise two AAV ITRs.

In a use or a method according to the invention, at least about 50%, at least about 60%, at least about 70% , at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, at least about 99% or higher of the AAV vector thus produced comprises AAV genome in the form of monomeric duplex.

In a use or a method of the invention, the insect cells may be infected with at least a first recombinant baculovirus harbouring an AAV vector genome and a second recombinant baculovirus harbouring a rep78/52 nucleotide sequence and wherein the m.o.i. of the recombinant baculovirus harbouring the rep78/52 nucleotide sequence is the higher.

In the use/method of the invention, expression of: Rep78 and/or Rep 68; and Rep 52 and/or Rep40 may be biphasic. In such a biphasic expression, the total level of Rep78 and Rep 68 protein expression is increased relative to the total level of Rep52 and Rep 40 protein expression in the first phase and the total level of Rep52 and Rep 40 protein expression is increased relative to the total level of Rep78 and Rep68 protein expression in the second phase.

Typically, total Rep52 and Rep40 expression may be increased relative to total Rep78 and Rep 68 expression during at least part of the encapsidation phase.

Preferably, the vector genome is smaller in length that a wild type AAV vector genome. For example, may be about half the size of a wild type AAV genome in length. The vector genome may be less than about 2.5 kb in length and result in the encapsidation of a concatamer of monomeric duplex genomes. For example, a genome may comprise one, two, three, four, five, six, seven, eight or more copies of the genome.

The use/method of the invention may result in the formation of AAV comprising a partial monomeric duplex genome. This may result if the genome vector has a length of greater than about 50% of the wild type AAV vector genome (and, typically, a length of less than about 100% of the wild type AAV vector genome.

Accordingly, the vector genome for producing the monomeric duplexed parvovirus virion DNA in the use/method of the invention is preferably about half of the size of the wild-type parvovirus genome (e.g., AAV) corresponding to the capsid into which the vDNA will be packaged. Alternatively, the template may be from about 40% to about 55% of wild type in length, such as from about more preferably from about 45% to about 52% of wt. Thus, the monomeric duplexed virion DNA produced from such a vector genome may have a total size that is approximately the size of the wild-type parvovirus genome (e.g., AAV) corresponding to the capsid into which the virion DNA will be packaged, e.g., from about 80% to about 105% of wt.

Alternatively stated, the vector genome will typically be less than about 2.5 kb in length (more preferably less than about 2.4 kb, still more preferably less than about 2.2 kb in length, yet more preferably less than about 2.1 kb in length) to facilitate packaging of the duplexed template by the parvovirus (e. g., AAV) capsid.

The template (as described above) is replicated to produce a duplexed vector genome (vDNA) of the invention, which is capable of forming a double-stranded DNA under appropriate conditions.

The monomeric duplexed parvoviruses of the present invention provide the host cell with a double-stranded molecule that addresses one of the drawbacks of rAAV vectors, i.e., the need for the host cell to convert the single-stranded rAAV vDNA into a double-stranded DNA.

The use/method of the invention may be such that a first promoter active in insect cells is used to drive the expression of rep78 and/or rep 68 and a second promoter active in insect cells is used to drive the expression of rep52 and/or rep68, wherein the activity of the first promoter is relatively weaker as compared with the activity of second promoter in said insect cells. Suitable promoters may be selected from any of those present in the baculovirus genome. Typically, but optionally, the weak promoter is selected from the early baculovirus promoters, in particular the dlE1 promoter and a strong promoter is typically a P10 or PolH promoter but the choice is not limited to this.

The nucleotide sequences encoding the Rep78/68 and Rep52/40 proteins may be present on the same or separate expression vectors.

The Rep78/68 and Rep 52/40 proteins may be encoded by and expressed from a single open reading frame.

The rep52/40 protein may be expressed from an internal promoter located in an intron sequence present in the rep78/68 encoding sequence in insect cells.

The invention also provides a method for the preparation of monomeric duplex containing AAV vector, said method comprising expressing in insect cells AAV Rep proteins and AAV Cap proteins in the presence of a vector genome comprising at least one AAV ITR, wherein said Rep proteins are expressed from a sequence as set out in any one of SEQ ID Nos: 1 to 7.

The invention further provides the use of an insect cell capable of expressing AAV Rep proteins and AAV Cap proteins in the presence of a vector genome comprising at least one AAV ITR, wherein total Rep52 and 40 protein expression is increased relative to total Rep78 and 68 protein expression in the preparation of monomeric duplex containing AAV vector.

The invention also relates to the use of an insect cell capable of expressing AAV Rep proteins and AAV Cap proteins in the presence of a vector genome comprising at least one AAV ITR, wherein the nicking activity of Rep78 is reduced relative to the helicase/encapsidation activity of Rep52 in the preparation of monomeric duplex containing AAV vector.

Also, the invention relation to the use of an insect cell capable of expressing AAV Rep proteins and AAV Cap proteins in the presence of a vector genome comprising at least one AAV ITR, wherein the Rep proteins are encoded by a nucleotide sequence as set out in any one of SEQ ID Nos: 1 to 7 in the preparation of monomeric duplex containing AAV vector.

The invention also concerns use of a Rep encoding sequence according to any one of SEQ ID Nos: 1 to 7 in the preparation of monomeric duplex containing AAV vector in insect cells.

A nucleotide sequence encoding animal parvoviruses Rep proteins, is herein understood as a nucleotide sequence encoding the non-structural Rep proteins that are required and sufficient for parvoviral vector production in insect cells such the Rep78 and Rep52 proteins, or the Rep68 and Rep40 proteins, or the combination of two or more thereof.

The animal parvovirus nucleotide sequence preferably is from a dependovirus, more preferably from a human or simian adeno-associated virus (AAV) and most preferably from an AAV which normally infects humans (e.g., serotypes 1, 2, 3A, 3B,

4, 5, and 6) or primates (e.g., serotypes 1 and 4). Rep coding sequences are well known to those skilled in the art and suitable sequences are referred to and described in detail in WO2007/148971 and also in WO2009/014445. Examples of rep coding sequences suitable for use in the invention are set out in SEQ ID NOs: 1 to 7. In addition, the construct referred to as VD183 (WO2009/014445) may be used in the invention. The rep52 and rep78 sequences used in VD183 are set out in SEQ ID NOs: 8 and 9 respectively. That construct uses the deltaE1 promoter to drive rep78 expression and the PolH promoter to drive rep52 expression. The AAV2 Rep 78 nucleic acid and protein sequences are set out in SEQ ID NOs: 10 and 11 respectively.

In the invention, a specified Rep protein is any peptide or protein sequence that has the activity characteristic to that specified Rep protein. That is to say, the Rep protein may have a wild-type sequence characteristic of a Rep protein, for example a wild-type Rep78 sequence. Alternatively, the Rep protein may have a non-wild type sequence, for example a variant Rep78 sequence, but which variant sequence retains one or more properties characteristic of a Rep78 sequence. This applies to any Rep sequence referred to herein. That is to say, reference to a Rep sequence includes all wild-type sequences (from any AAV serotype) and any variant sequence retains one or more properties characteristic of a Rep78 sequence. This applies in particular to references to Rep78, Rep 68, Rep 52 and Rep 40 herein. In addition reference to a Rep protein from AAV also encompasses functional fragments of such a Rep protein and functionally corresponding Rep sequences from other parvoviral viruses.

Preferably, the nucleotide sequence encodes animal parvoviruses Rep proteins that are required and sufficient for parvoviral vector production in insect cells.

Elimination of possible false translation initiation sites in the Rep protein coding sequences, other than the Rep78 and Rep52 translation initiation sites, of other parvoviruses will be well understood by an artisan of skill in the art, as will be the elimination of putative splice sites that may be recognised in insect cells. The various modifications of the wild-type parvoviral sequences for proper expression in insect cells is achieved by application of well-known genetic engineering techniques such as described e.g. in Sambrook and Russell (2001) "Molecular Cloning: A Laboratory Manual (3rd edition), Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, New York. Various further modifications of Rep protein coding regions are

known to the skilled artisan which could increase yield of Rep protein. These modifications are within the scope of the present invention.

In, the invention a nucleotide sequence encoding a parvoviral Rep protein(s) may be as defined above and typically used in the form of a nucleic acid construct. Preferably, in the construct, the nucleotide sequence encoding the parvoviral Rep proteins is operably linked to expression control sequences for expression in an insect cell. These expression control sequences will at least include a promoter that is active in insect cells. Techniques known to one skilled in the art for expressing foreign genes in insect host cells can be used to practice the invention. Methodology for molecular engineering and expression of polypeptides in insect cells is described, for example, in Summers and Smith. 1986. A Manual of Methods for Baculovirus Vectors and Insect Culture Procedures, Texas Agricultural Experimental Station Bull. No. 7555, College Station, Tex.; Luckow. 1991. In Prokop et al., Cloning and Expression of Heterologous Genes in Insect Cells with Baculovirus Vectors' Recombinant DNA Technology and Applications, 97-152; King, L. A. and R. D. Possee, 1992, The baculovirus expression system, Chapman and Hall, United Kingdom; O'Reilly, D. R., L. K. Miller, V. A. Luckow, 1992, Baculovirus Expression Vectors: A Laboratory Manual, New York; W. H. Freeman and Richardson, C. D., 1995, Baculovirus Expression Protocols, Methods in Molecular Biology, volume 39; US 4,745,051; US2003148506; and WO 03/074714. A particularly suitable promoter for transcription of the nucleotide sequence of the invention encoding of the parvoviral Rep proteins is e.g. the polyhedron promoter. However, other promoters that are active in insect cells are known in the art, e.g. the p10, p35, IE-1 or ΔIE-1 promoters and further promoters described in the above references.

Preferably the nucleic acid construct for expression of the parvoviral Rep proteins in insect cells is an insect cell-compatible vector. An "insect cell-compatible vector" or "vector" is understood to a nucleic acid molecule capable of productive transformation or transfection of an insect or insect cell. Exemplary biological vectors include plasmids, linear nucleic acid molecules, and recombinant viruses. Any vector can be employed as long as it is insect cell-compatible. The vector may integrate into the insect cells genome but the presence of the vector in the insect cell need not be permanent and transient episomal vectors are also included. The vectors can be introduced by any means known, for example by chemical treatment of the cells,

electroporation, or infection. In a preferred embodiment, the vector is a baculovirus, a viral vector, or a plasmid. In a more preferred embodiment, the vector is a baculovirus, i.e. the construct is a baculoviral vector. Baculoviral vectors and methods for their use are described in the above cited references on molecular engineering of insect cells.

5 Any insect cell which allows for replication of a recombinant parvoviral (rAAV) vector and which can be maintained in culture can be used in accordance with the present invention. For example, the cell line used can be from Spodoptera frugiperda, drosophila cell lines, or mosquito cell lines, e.g., Aedes albopictus derived cell lines. Preferred insect cells or cell lines are cells from the insect species which are susceptible 10 to baculovirus infection, including e.g. Se301, SeIZD2109, SeUCR1, Sf9, Sf900+, Sf21, BTI-TN-5B1-4, MG-1, Tn368, HzAm1, Ha2302, Hz2E5, High Five (Invitrogen, CA, USA) and *expres*SF+® (US 6,103,526; Protein Sciences Corp., CT, USA).

In the context of the invention "at least one parvoviral ITR nucleotide sequence" is understood to mean a palindromic sequence, comprising mostly complementary, 15 symmetrically arranged sequences also referred to as "A," "B," and "C" regions. The ITR functions as an origin of replication, a site having a "cis" role in replication, i.e., being a recognition site for trans acting replication proteins such as e.g. Rep 78 (or Rep68) which recognize the palindrome and specific sequences internal to the palindrome. One exception to the symmetry of the ITR sequence is the "D" region of 20 the ITR. It is unique (not having a complement within one ITR). Nicking of single-stranded DNA occurs at the junction between the A and D regions. It is the region where new DNA synthesis initiates. The D region normally sits to one side of the palindrome and provides directionality to the nucleic acid replication step. An parvovirus replicating in a mammalian cell typically has two ITR sequences. It is, 25 however, possible to engineer an ITR so that binding sites are on both strands of the A regions and D regions are located symmetrically, one on each side of the palindrome. On a double-stranded circular DNA template (e.g., a plasmid), the Rep78- or Rep68-assisted nucleic acid replication then proceeds in both directions and a single ITR suffices for parvoviral replication of a circular vector. Thus, one ITR nucleotide 30 sequence can be used in the context of the present invention. Preferably, however, two or another even number of regular ITRs are used. Most preferably, two ITR sequences are used. A preferred parvoviral ITR is an AAV ITR. For safety reasons it may be desirable to construct a recombinant parvoviral (rAAV) vector that is unable to further

propagate after initial introduction into a cell. Such a safety mechanism for limiting undesirable vector propagation in a recipient may be provided by using rAAV with a chimeric ITR as described in US2003148506.

Those skilled in the art will appreciate that the viral Rep protein(s) used for 5 producing the monomeric duplexed vectors according to the invention may be selected with consideration for the source of the viral ITRs. For example, the AAV5 ITR typically interacts more efficiently with the AAV5 Rep protein, although it is not necessary that the serotype of ITR and Rep protein(s) are matched.

The ITR(s) used in the invention are typically functional, i.e. they may be fully 10 resolvable and are preferably AAV sequences, with serotypes 1, 2, 3, 4, 5 and 6 being preferred. Resolvable AAV ITRs according to the present invention need not have a wild-type ITR sequence (e. g., a wild-type sequence may be altered by insertion, deletion, truncation or missense mutations), as long as the ITR mediates the desired functions, e. g., virus packaging, integration, and/or provirus rescue, and the like.

15 In a preferred embodiment of the invention, the vector genome, typically a sequence comprising at least one parvoviral (AAV) ITR, further comprises at least one nucleotide sequence encoding a gene product of interest, whereby preferably the at least one nucleotide sequence encoding a gene product of interest becomes incorporated into the genome of a recombinant parvoviral (rAAV) vector produced in 20 an insect cell. Preferably, at least one nucleotide sequence encoding a gene product of interest is a sequence for expression in a mammalian cell. Preferably, the vector genome comprises two parvoviral (AAV) ITR nucleotide sequences and wherein the at least one nucleotide sequence encoding a gene product of interest is located between the two parvoviral (AAV) ITR nucleotide sequences. Preferably, the nucleotide 25 sequence encoding a gene product of interest (for expression in a target cell) will be incorporated into the recombinant parvoviral (rAAV) vector produced in the insect cell if it is located between two regular ITRs, or is located on either side of an ITR engineered with two D regions.

AAV sequences that may be used in the present invention for the production of 30 recombinant AAV vectors in insect cells can be derived from the genome of any AAV serotype. Generally, the AAV serotypes have genomic sequences of significant homology at the amino acid and the nucleic acid levels, provide an identical set of genetic functions, produce virions which are essentially physically and functionally

equivalent, and replicate and assemble by practically identical mechanisms. For the genomic sequence of the various AAV serotypes and an overview of the genomic similarities see e.g. GenBank Accession number U89790; GenBank Accession number J01901; GenBank Accession number AF043303; GenBank Accession number
5 AF085716; Chlorini et al. (1997, J. Vir. 71: 6823-33); Srivastava et al. (1983, J. Vir. 45:555-64); Chlorini et al. (1999, J. Vir. 73:1309-1319); Rutledge et al. (1998, J. Vir. 72:309-319); and Wu et al. (2000, J. Vir. 74: 8635-47). AAV serotypes 1, 2, 3, 4 and 5 are preferred source of AAV nucleotide sequences for use in the context of the present invention. Preferably the AAV ITR sequences for use in the context of the present
10 invention are derived from AAV1, AAV2, and/or AAV4. Likewise, the Rep (Rep78, Rep52, Rep 68 and Rep 40) coding sequences are preferably derived from AAV1, AAV2, and/or AAV4. The sequences coding for the VP1, VP2, and VP3 capsid proteins for use in the context of the present invention may however be taken from any of the known 42 serotypes, more preferably from AAV1, AAV2, AAV3, AAV4,
15 AAV5, AAV6, AAV7, AAV8 or AAV9 or newly developed AAV-like particles obtained by e.g. capsid shuffling techniques and AAV capsid libraries.

AAV Rep and ITR sequences are particularly conserved among most serotypes. The Rep78 proteins of various AAV serotypes are e.g. more than 89% identical and the total nucleotide sequence identity at the genome level between AAV2, AAV3A,
20 AAV3B, and AAV6 is around 82% (Bantel-Schaal et al., 1999, J. Virol., 73(2):939-947). Moreover, the Rep sequences and ITRs of many AAV serotypes are known to efficiently cross-complement (i.e., functionally substitute) corresponding sequences from other serotypes in production of AAV particles in mammalian cells. US2003148506 reports that AAV Rep and ITR sequences also efficiently cross-
25 complement other AAV Rep and ITR sequences in insect cells.

The AAV VP proteins are known to determine the cellular tropicity of the AAV virion. The VP protein-encoding sequences are significantly less conserved than Rep proteins and genes among different AAV serotypes. The ability of Rep and ITR sequences to cross-complement corresponding sequences of other serotypes allows for
30 the production of pseudotyped rAAV particles comprising the capsid proteins of a serotype (e.g., AAV3) and the Rep and/or ITR sequences of another AAV serotype (e.g., AAV2). Such pseudotyped rAAV particles are a part of the present invention.

Modified "AAV" sequences also can be used in the context of the present invention, e.g. for the production of rAAV vectors in insect cells. Such modified sequences e.g. include sequences having at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more
5 nucleotide and/or amino acid sequence identity (e.g., a sequence having about 75-99% nucleotide sequence identity) to an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8 or AAV9 ITR, Rep, or VP can be used in place of wild-type AAV ITR, Rep, or VP sequences.

Although similar to other AAV serotypes in many respects, AAV5 differs from
10 other human and simian AAV serotypes more than other known human and simian serotypes. In view thereof, the production of rAAV5 can differ from production of other serotypes in insect cells. Where methods of the invention are employed to produce rAAV5, it is preferred that one or more constructs comprising, collectively in the case of more than one construct, a nucleotide sequence comprising an AAV5 ITR, a
15 nucleotide sequence comprises an AAV5 Rep coding sequence (i.e. a nucleotide sequence comprises an AAV5 Rep78). Such ITR and Rep sequences can be modified as desired to obtain efficient production of rAAV5 or pseudotyped rAAV5 vectors in insect cells. E.g., the start codon of the Rep sequences can be modified, VP splice sites can be modified or eliminated, and/or the VP1 start codon and nearby nucleotides can
20 be modified to improve the production of rAAV5 vectors in the insect cell.

Thus, the viral capsid used in the invention may be from any parvovirus, either an autonomous parvovirus or dependovirus, as described above. Preferably, the viral capsid is an AAV capsid (e. g., AAV1, AAV2, AAV3, AAV4, AAV5 or AAV6 capsid). In general, the AAV1 capsid, AAV5 capsid, and AAV3 capsid are preferred.
25 The choice of parvovirus capsid may be based on a number of considerations as known in the art, e. g., the target cell type, the desired level of expression, the nature of the heterologous nucleotide sequence to be expressed, issues related to viral production, and the like. For example, the AAV1 capsid may be advantageously employed for skeletal muscle, liver and cells of the central nervous system (e. g., brain); AAV5 for
30 cells in the airway and lung ; AAV3 for bone marrow cells ; and AAV4 for particular cells in the brain (e. g., appendable cells).

The vector genome typically comprises a nucleotide sequence encoding at least one "gene product of interest", generally for expression in a mammalian cell, located

such that it will be incorporated into an rAAV monomeric duplex vector replicated in the insect cell. Any nucleotide sequence can be incorporated for later expression in a mammalian cell transfected with the recombinant parvoviral (rAAV) vector produced in accordance with the present invention. The nucleotide sequence may e.g. encode a protein or it may express an RNAi agent, i.e. an RNA molecule that is capable of RNA interference such as e.g. a shRNA (short hairpinRNA) or an siRNA (short interfering RNA). "siRNA" means a small interfering RNA that is a short-length double-stranded RNA that are not toxic in mammalian cells (Elbashir et al., 2001, Nature 411: 494-98; Caplen et al., 2001, Proc. Natl. Acad. Sci. USA 98: 9742-47). A vector genome used in the invention may comprise two nucleotide sequences and each encodes one gene product of interest for expression in a mammalian cell. Each of the two nucleotide sequences encoding a product of interest is located such that it will be incorporated into an rAAV monomeric duplex vector replicated in an insect cell.

The product of interest for expression in a mammalian cell may be a therapeutic gene product. A therapeutic gene product can be a polypeptide, or an RNA molecule (siRNA), or other gene product that, when expressed in a target cell, provides a desired therapeutic effect such as e.g. ablation of an undesired activity, e.g. the ablation of an infected cell, or the complementation of a genetic defect, e.g. causing a deficiency in an enzymatic activity. Examples of therapeutic polypeptide gene products include CFTR, Factor IX, Lipoprotein lipase (LPL, preferably LPL S447X; see WO 01/00220), Apolipoprotein A1, Uridine Diphosphate Glucuronosyltransferase (UGT), Retinitis Pigmentosa GTPase Regulator Interacting Protein (RP-GRIP), and cytokines or interleukins like e.g. IL-10.

Alternatively, or in addition as a second gene product, a vector genome sequence may comprise a nucleotide sequence encoding a polypeptide that serves as a marker proteins to assess cell transformation and expression. Suitable marker proteins for this purpose are e.g. the fluorescent protein GFP, and the selectable marker genes HSV thymidine kinase (for selection on HAT medium), bacterial hygromycin B phosphotransferase (for selection on hygromycin B), Tn5 aminoglycoside phosphotransferase (for selection on G418), and dihydrofolate reductase (DHFR) (for selection on methotrexate), CD20, the low affinity nerve growth factor gene. Sources for obtaining these marker genes and methods for their use are provided in Sambrook and Russel (2001) "Molecular Cloning: A Laboratory Manual (3rd edition), Cold Spring

Harbor Laboratory, Cold Spring Harbor Laboratory Press, New York. Furthermore, second nucleotide sequence defined herein above may comprise a nucleotide sequence encoding a polypeptide that may serve as a fail-safe mechanism that allows to cure a subject from cells transduced with the recombinant parvoviral (rAAV) vector of the invention, if deemed necessary. Such a nucleotide sequence, often referred to as a suicide gene, encodes a protein that is capable of converting a prodrug into a toxic substance that is capable of killing the transgenic cells in which the protein is expressed. Suitable examples of such suicide genes include e.g. the *E.coli* cytosine deaminase gene or one of the thymidine kinase genes from Herpes Simplex Virus, Cytomegalovirus and Varicella-Zoster virus, in which case ganciclovir may be used as prodrug to kill the transgenic cells in the subject (see e.g. Clair et al., 1987, Antimicrob. Agents Chemother. 31: 844-849).

In another embodiment, one of the gene products of interest can be an AAV protein. In particular, a Rep protein, such as Rep78 or Rep68, or a functional fragment thereof. A nucleotide sequence encoding a Rep78 and/or a Rep68, if present on the genome of a recombinant parvoviral (rAAV) vector of the invention and expressed in a mammalian cell transduced with the vector, allows for integration of the recombinant rAAV vector into the genome of the transduced mammalian cell. Expression of Rep78 and/or Rep68 in an rAAV-transduced or infected mammalian cell can provide an advantage for certain uses of the recombinant parvoviral (rAAV) vector, by allowing long term or permanent expression of any other gene product of interest introduced in the cell by the vector.

In the recombinant parvoviral (rAAV) vectors of the invention, at least one nucleotide sequence(s) encoding a gene product of interest for expression in a mammalian cell, preferably is/are operably linked to at least one mammalian cell-compatible expression control sequence, e.g., a promoter. Many such promoters are known in the art (see Sambrook and Russel, 2001, *supra*). Contitutive promoters that are broadly expressed in many cell-types, such as the CMV promoter may be used. However, more preferred will be promoters that are inducible, tissue-specific, cell-type-specific, or cell cycle-specific. For example, for liver-specific expression a promoter may be selected from an α1-anti-trypsin promoter, a thyroid hormone-binding globulin promoter, an albumin promoter, LPS (thyroxine-binding globlin) promoter, HCR-ApoCII hybrid promoter, HCR-hAAT hybrid promoter and an apolipoprotein E

promoter. Other examples include the E2F promoter for tumor-selective, and, in particular, neurological cell tumor-selective expression (Parr et al., 1997, Nat. Med. 3:1145-9) or the IL-2 promoter for use in mononuclear blood cells (Hagenbaugh et al., 1997, J Exp Med; 185: 2101-10).

5 A method of the invention may preferably comprise the step of affinity-purification of the (virions comprising the) recombinant parvoviral (rAAV) vector using an anti-AAV antibody, preferably an immobilised antibody. The anti-AAV antibody preferably is an monoclonal antibody. A particularly suitable antibody is a single chain camelid antibody or a fragment thereof as e.g. obtainable from camels or 10 llamas (see e.g. Muyldermans, 2001, Biotechnol. 74: 277-302). The antibody for affinity-purification of rAAV preferably is an antibody that specifically binds an epitope on a AAV capsid protein, whereby preferably the epitope is an epitope that is present on capsid protein of more than one AAV serotype. E.g. the antibody may be raised or selected on the basis of specific binding to AAV2 capsid but at the same time 15 also it may also specifically bind to AAV1, AAV3 and AAV5 capsids.

Accordingly, the invention relates to a monomeric duplex comprising AAV vector produced and/or produceable by a use or method of the invention as described herein.

The use or method of the invention may result in the formation of AAV 20 comprising a partial monomeric duplex genome.

The parvovirus particles prepared according to the invention may be a "hybrid" particle in which the viral TRs and viral capsid are from different parvoviruses. Preferably, the viral TRs and capsid are from different serotypes of AAV. Likewise, the parvovirus may have a "chimeric" capsid (e. g., containing sequences from different 25 parvoviruses, preferably different AAV serotypes) or a "targeted" capsid (e. g., a directed tropism).

As used herein, a"duplexed parvovirus particle" encompasses hybrid, chimeric and targeted virus particles. Preferably, the duplexed parvovirus particle has an AAV capsid, which may further by a chimeric or targeted capsid, as described above.

30 Preferably, the monomeric duplexed parvovirus particle prepared according to the method of the invention has an AAV capsid, which may further by a chimeric or targeted capsid, as described above.

The monomeric duplexed parvovirus particles of the invention comprise a parvovirus capsid containing a virion DNA (vDNA). The vDNA is self-complementary so that it may form a hairpin structure upon release from the viral capsid. The duplexed vDNA appears to provide to the host cell a double-stranded DNA that may be expressed (i. e., transcribed and, optionally, translated) by the host cell without the need for second-strand synthesis, as required with conventional parvovirus vectors.

The duplexed parvovirus vector genome preferably contains sufficient packaging sequences for encapsidation within the selected parvovirus capsid (e. g, AAV capsid).

Those skilled in the art will appreciate that the duplexed vDNA may not exist in a double-stranded form under all conditions, but has the ability to do so under conditions that favor annealing of complementary nucleotide bases.

Accordingly, the term "monomeric duplexed parvovirus vector" or the like does not indicate that the vDNA is necessarily in duplexed or double-stranded form (e. g., there is base-pairing between the self-complementary strands) within the parvovirus capsid itself.

Indeed, one skilled in the art will understand that the vDNA may not be in a double-stranded form while packaged within the parvovirus capsid.

It seems likely that the virion genome is retained in a single-stranded form while packaged within the viral capsid. Upon release from the capsid during viral infection, it appears that the dimeric molecule "snaps back" or anneals to form a double-stranded molecule by intra-strand basepairing, with the ITR sequence forming a covalently-closed hairpin structure at one end. This double-stranded vDNA obviates host cell mediated second-strand synthesis, which has been postulated to be a rate-limiting step for AAV transduction.

A composition comprising AAV vector of which at least about 50%, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, at least about 99% or higher is in the form of monomeric duplex AAV is provided according to the invention. That is to say, it is AAV vector which comprises a vector genome in the form of a monomeric duplex.

The reagents, uses and methods disclosed herein may be employed to produce high-titer stocks of the monomeric duplex AAV vectors, preferably at essentially wild-type titers. It is also preferred that the parvovirus stock has a titer of at least about 10^6

genome copies (gc)/ml, at least about 10^7 gc/ml, at least about 10^8 gc/ml, at least about 10^9 gc/ml, at least about 10^{10} gc/ml, at least about 10^{11} gc/ml, at least about 10^{12} gc/ml, at least about 10^{13} gc/ml, at least about 10^{14} gc/ml, at least about 10^{15} gc/ml, at least about 10^{16} gc/ml or higher.

5 Further, the monomeric duplexed parvovirus vectors prepared according to the invention, may have an improved genome copy (gc)/particle ratio over conventional parvovirus vectors. Preferably, the tu/particle ratio is less than about 50:1, less than about 20:1, less than about 15:1, less than about 10:1, less than about 8:1, less than about 7:1, less than about 6:1, less than about 5:1, less than about 4:1, or lower. There 10 is no particular lower limit to the gc/particle ratio.

Expression of a heterologous nucleotide sequence (as described below) is preferably "enhanced" from the monomeric duplexed parvovirus vectors of the invention as compared with the comparable parvovirus (e. g., rAAV) vector.

Preferably, gene expression may be detected from the monomeric duplexed 15 parvovirus vector substantially more rapidly than from the comparable monomeric parvovirus vector. For example, gene expression may be detected in less than about 2 weeks, preferably less than about one week, more preferably less than about 72 hours, still more preferably less than about 48 hours, and still more preferably less than about 24 hours after administration of the duplexed parvovirus vector. Gene expression may 20 be detected by any method known in the art, e. g., by detecting transcription, translation, or biological activity or a phenotypic effect resulting from expression of a heterologous nucleotide sequence (e. g., blood clotting time).

Alternatively, gene expression from the monomeric duplexed parvovirus vector may be "enhanced" in that higher levels of gene expression (as defined in the preceding 25 paragraph) are detected as compared with the comparable monomeric parvovirus vector (e. g., rAAV vector). Comparisons may be made in the level of gene expression at the same time point after administration of virus. Alternatively, comparisons may be made between the maximum level of gene expression achieved with each vector.

Such a composition may be combined with an pharmaceutically acceptable 30 carrier or diluent to give to a pharmaceutical composition of the invention.

The AAV vector produced according to the invention may be of use in transferring genetic material to a cell. Such transfer may take place *in vitro*, *ex vivo* or *in vivo*.

Monomeric duplexed parvovirus vectors (as prepared according to the method of the invention) can be advantageously employed for gene delivery. These monomeric duplexed parvovirus vectors may be more efficient than single stranded AAV vectors, e. g., improved transduction to particle ratios, more rapid transgene expression, a higher level of transgene expression, and/or more persistent transgene expression. The vectors prepared according to the invention may be used for gene delivery to host cells that are typically refractory to AAV transduction. Thus, these duplexed parvovirus vectors have a different (e. g., broader) host range than do AAV vectors.

Accordingly, the invention comprises a method for delivering a nucleotide sequence to a cell, which method comprises contacting a composition or a pharmaceutical composition as described herein under conditions such that the monomeric duplex containing AAV enters the cell.

The invention also provides a method for administering a nucleotide sequence to a subject, which method comprises adminstering to the said subject a composition or a pharmaceutical composition as described herein.

Accordingly, the invention further provides use of a composition as described herein in the manufacture of a medicament for use in the administration of nucleotide to a subject.

The present invention further provides methods of producing and administering the monomeric duplexed parvovirus vectors of the invention. In one particular embodiment, the present invention provides a method of administering a nucleotide sequence to a subject, comprising administering to a subject a monomeric duplexed parvovirus particle according to the invention together with a pharmaceutically acceptable carrier. Preferably, the duplexed parvovirus particle is administered in a therapeutically-effective amount to a subject in need thereof.

The vector, composition or pharmaceutical composition may be delivered to a cell in vitro or to a subject in vivo by any suitable method known in the art. Alternatively, the vector may be delivered to a cell ex vivo, and the cell administered to a subject, as known in the art.

Such methods may be advantageously employed to provide more efficient transduction of target cells than wild type AAV vectors. To illustrate, the duplexed parvovirus vectors may transduce at a higher rate than wt AAV vectors. Alternatively, or additionally, the duplexed parvovirus vectors may provide for a more rapid onset of

transgene expression, a higher level of transgene expression, and/or a longer persistence of transgene expression than AAV vectors.

The vector, composition or pharmaceutical composition may further find use in methods of administering a nucleotide sequence to a cell that is typically non-permissive for transduction by AAV, or is only inefficiently transduced by AAV. Exemplary cells include but are not limited to dendritic cells, particular types of cancer or tumor cells, astrocytes, and bone marrow stem cells. Moreover, the methods disclosed herein may be advantageously practiced with non-replicating or slowly-replicating cells that only inefficiently support second-strand AAV synthesis, such as the liver, central nervous system (e. g., brain), and particular populations of cells within muscle (e. g., fast-twitch fibers).

Accordingly, the vector, composition or pharmaceutical composition disclosed herein may have a distinct target cell range (e. g., a broader range of target cells) as compared with rAAV vectors. While not wishing to be held to any particular theory of the invention, it appears that cells that are refractory to transduction by rAAV may be permissive for the monomeric duplexed vectors described herein, which provide a double-stranded molecule to the host cell. Thus, the present invention finds use for delivering a nucleotide sequence to a cell that is non-permissive for conventional rAAV vectors or only poorly transduced by rAAV vectors because it cannot efficiently support second-strand synthesis of the viral DNA.

The present invention may be used to deliver a heterologous nucleotide sequence. The more rapid onset of transgene expression by a monomeric duplexed parvovirus vector may make these gene delivery vehicles more amenable to such treatment regimes than are rAAV vectors.

Any heterologous nucleotide sequence(s) may be delivered according to the present invention. Nucleic acids of interest include nucleic acids encoding polypeptides, preferably therapeutic (e. g., for medical or veterinary uses) or immunogenic (e. g., for vaccines) polypeptides.

A "therapeutic polypeptide" is a polypeptide that may alleviate or reduce symptoms that result from an absence or defect in a protein in a cell or subject. Alternatively, a"therapeutic polypeptide"is one that otherwise confers a benefit to a subject, e. g., anti-cancer effects or improvement in transplant survivability.

Preferably, the heterologous nucleotide sequence or sequences will be less than about 2.5 kb in length (more preferably less than about 2.4 kb, still more preferably less than about 2.2 kb, yet more preferably less than about 2.0 kb in length) to facilitate packaging of the monomeric duplexed template by the parvovirus (e. g., AAV) capsid.

5 Exemplary nucleotide sequences encode Factor VIII, IX, Factor X, lysosomal enzymes (e. g., hexosaminidase A, associated with Tay-Sachs disease, or iduronate sulfatase, associated with Hunter Syndrome/MPS il), erythropoietin, angiostatin, endostatin, superoxide dismutase, globin, leptin, catalase, tyrosine hydroxylase, as well as cytokines (e. g., a-interferon, -interferon, interferon-y, interleukin-2, interleukin-4,
10 interleukin 12, granulocyte-macrophage colony stimulating factor, lymphotoxin, and the like), peptide growth factors and hormones (e. g., somatotropin, insulin, insulin-like growth factors 1 and 2, platelet derived growth factor, epidermal growth factor, fibroblast growth factor, nerve growth factor, neurotrophic factor-3 and-4, brain-derived neurotrophic factor, glial derived growth factor, transforming growth factor-a
15 and-j3, and the like), receptors (e. g., tumor necrosis factor receptor). In other exemplary embodiments, the heterologous nucleotide sequence encodes a monoclonal antibodies, preferably a single-chained monoclonal antibody or a monoclonal antibody directed against a cancer or tumor antigen (e. g., HER2/neu, and as described below). Other illustrative heterologous nucleotide sequences encode suicide gene products
20 (thymidine kinase, cytosine deaminase, diphtheria toxin, cytochrome P450, deoxycytidine kinase, and tumor necrosis factor), proteins conferring resistance to a drug used in cancer therapy, and tumor suppressor gene products.

As a further alternative, the heterologous nucleic acid sequence may encode a reporter polypeptide (e. g., an enzyme such as Green Fluorescent
25 Protein, alkaline phosphatase).

Alternatively, in particular embodiments of the invention, the nucleic acid of interest may encode an antisense nucleic acid, a ribozyme, RNAs that effect spliceosome mediated trans-splicing, interfering RNAs (RNAi) that mediate gene silencing or other non-translated RNAs, such as "guide" RNAs and the like.

30 The monomeric duplexed vector may also encode a heterologous nucleotide sequence that shares homology with and recombines with a locus on the host chromosome. This approach may be utilized to correct a genetic defect in the host cell.

The present invention may also be used to express an immunogenic polypeptide in a subject, e. g., for vaccination. The nucleic acid may encode any immunogen of interest known in the art including, but are not limited to, immunogens from human immunodeficiency virus, influenza virus, gag proteins, tumor antigens, cancer antigens, bacterial antigens, viral antigens, and the like.

Alternatively, the heterologous nucleotide sequence may encode any polypeptide that is desirably produced in a cell *in vitro*, *ex vivo*, or *in vivo*. For example, the monomeric duplexed vectors may be introduced into cultured cells and the expressed gene product isolated therefrom.

It will be understood by those skilled in the art that the heterologous nucleotide sequence (s) of interest may be operably associated with appropriate control sequences. For example, the heterologous nucleic acid may be operably associated with expression control elements, such as transcription/translation control signals, origins of replication, polyadenylation signals, and internal ribosome entry sites (IRES), promoters, enhancers, and the like.

Those skilled in the art will appreciate that a variety of promoter/enhancer elements may be used depending on the level and tissuespecific expression desired. The promoter/enhancer may be constitutive or inducible, depending on the pattern of expression desired. The promoter/enhancer may be native or foreign and can be a natural or a synthetic sequence. By foreign, it is intended that the transcriptional initiation region is not found in the wild-type host into which the transcriptional initiation region is introduced.

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

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

initiation codon and adjacent sequences, can be of a variety of origins, both natural and synthetic.

The methods of the present invention also provide a means for delivering heterologous nucleotide sequences into a broad range of cells, including dividing and
5 non-dividing cells. The present invention may be employed to deliver a nucleotide sequence of interest to a cell *in vitro*, e. g., to produce a polypeptide *in vitro* or for *ex vivo* gene therapy. The cells, pharmaceutical formulations, and methods of the present invention are additionally useful in a method of delivering a nucleotide sequence to a subject in need thereof, e. g., to express an immunogenic or therapeutic polypeptide. In
10 this manner, the polypeptide may thus be produced in vivo in the subject. The subject may be in need of the polypeptide because the subject has a deficiency of the polypeptide, or because the production of the polypeptide in the subject may impart some therapeutic effect, as a method of treatment or otherwise, and as explained further below.

15 In general, the present invention may be employed to deliver any foreign nucleic acid with a biological effect to treat or ameliorate the symptoms associated with any disorder related to gene expression.

Gene transfer has substantial potential use in understanding and providing therapy for disease states. There are a number of inherited diseases in which defective
20 genes are known and have been cloned. In general, the above disease states fall into two classes: deficiency states, usually of enzymes, which are generally inherited in a recessive manner, and unbalanced states, which may involve regulator or structural proteins, and which are typically inherited in a dominant manner. For deficiency state diseases, gene transfer could be used to bring a normal gene into affected tissues for
25 replacement therapy, as well as to create animal models for the disease using antisense mutations. For unbalanced disease states, gene transfer could be used to create a disease state in a model system, which could then be used in efforts to counteract the disease state. Thus the methods of the present invention permit the treatment of genetic diseases. As used herein, a disease state is treated by partially or wholly remedying the
30 deficiency or imbalance that causes the disease or makes it more severe.

The instant invention may also be employed to provide an antisense nucleic acid to a cell in vitro or in vivo. Expression of the antisense nucleic acid in the target cell diminishes expression of a particular protein by the cell.

Accordingly, antisense nucleic acids may be administered to decrease expression of a particular protein in a subject in need thereof. Antisense nucleic acids may also be administered to cells in vitro to regulate cell physiology, e. g., to optimize cell or tissue culture systems.

5 In general, the present invention can be employed to deliver any heterologous nucleic acid to a cell *in vitro*, *ex vivo*, or *in vivo*.

The present invention finds use in both veterinary and medical applications. Suitable subjects for *ex vivo* gene delivery methods as described above include both avians and mammals, with mammals being preferred. The term "avian" as used herein 10 includes, but is not limited to, chickens, ducks, geese, quail, turkeys and pheasants. The term "mammal" as used herein includes, but is not limited to, humans, bovines, ovines, caprines, equines, felines, canines, lagomorphs, etc. Human subjects are most preferred. Human subjects include neonates, infants, juveniles, and adults.

In particular embodiments, the present invention provides a pharmaceutical 15 composition comprising a monomeric duplex vector of the invention in a pharmaceutically-acceptable carrier and/or other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, etc. For injection, the carrier will typically be a liquid. For other methods of administration, the carrier may be either solid or liquid. For inhalation administration, the carrier will be respirable, and will preferably be in 20 solid or liquid particulate form. As an injection medium, it is preferred to use water that contains the additives usual for injection solutions, such as stabilizing agents, salts or saline, and/or buffers.

In general, a "physiologically acceptable carrier" is one that is not toxic or unduly detrimental to cells. Exemplary physiologically acceptable carriers include sterile, 25 pyrogen-free water and sterile, pyrogen-free, phosphate buffered saline. Physiologically-acceptable carriers include pharmaceuticallyacceptable carriers.

By "pharmaceutically acceptable" it is meant a material that is not biologically or otherwise undesirable, i. e, the material may be administered to a subject without causing any undesirable biological effects. Thus, such a pharmaceutical composition 30 may be used, for example, in transfection of a cell *ex vivo* or in administering a viral particle or cell directly to a subject.

The parvovirus vectors of the invention maybe administered to elicit an immunogenic response (e. g., as a vaccine). Typically, vaccines of the present invention

comprise an immunogenic amount of infectious virus particles as disclosed herein in combination with a pharmaceutically acceptable carrier. An "immunogenic amount" is an amount of the infectious virus particles that is sufficient to evoke an immune response in the subject to which the pharmaceutical formulation is administered.

5 The present invention further provides a method of delivering a nucleic acid to a cell. Typically, for *in vitro* methods, the virus may be introduced into the cell by standard viral transduction methods, as are known in the art.

Preferably, the virus particles are added to the cells at the appropriate multiplicity of infection according to standard transduction methods appropriate for the particular
10 target cells. Titers of virus to administer can vary, depending upon the target cell type and the particular virus vector, and may be determined by those of skill in the art without undue experimentation.

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

20 The cell to be administered the monomeric duplex vector of the invention may be of any type, including but not limited to neural cells (including cells of the peripheral and central nervous systems, in particular, brain cells), lung cells, retinal cells, epithelial cells (e. g., gut and respiratory epithelial cells), muscle cells, dendritic cells, pancreatic cells (including islet cells), hepatic cells, myocardial cells, bone cells (e. g.,
25 bone marrow stem cells), hematopoietic stem cells, spleen cells, keratinocytes, fibroblasts, endothelial cells, prostate cells, germ cells, and the like. Alternatively, the cell may be any progenitor cell. As a further alternative, the cell can be a stem cell (e. g., neural stem cell, liver stem cell).

In particular embodiments of the invention, cells are removed from a subject, the
30 parvovirus vector is introduced therein, and the cells are then replaced back into the subject. Methods of removing cells from subject for treatment ex vivo, followed by introduction back into the subject are known in the art. Alternatively, the rAAV vector

is introduced into cells from another subject, into cultured cells, or into cells from any other suitable source, and the cells are administered to a subject in need thereof.

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

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

The parvovirus vector administered to the subject may transduce any permissive cell or tissue. Suitable cells for transduction by the monomeric duplex parvovirus vectors are as described above.

Dosages of the monomeric duplex parvovirus particles will depend upon the mode of administration, the disease or condition to be treated, the individual subject's condition, the particular virus vector, and the gene to be delivered, and can be determined in a routine manner. Typically, an amount of about 10^3 to about 10^{16} virus particles per dose may be suitable.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention.

All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition, reference to an element

by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

5 The following Examples illustrate the invention:

Examples

Materials and Methods:

rAAV production,

5 rAAV were produced by co infection with combinations of three different baculoviruses. Namely, Rep encoding baculovirus: Bac.VD88, Bac.VD183, Bac.VD195, Bac.VD196, Bac.VD197, Bac.VD210, Bac.VD216, Bac.VD217 FBDSLR; transgene encoding baculovirus: Bac.VD179 (seap), Bac.VD154 (aat-luc) Bac.VD263 (Reporter Gene 2), Bac.VD259 (mU1) and cap encoding baculovirus:
10 Bac.VD92 (cap5), Bac.VD254 (cap6). Varying ratio of Rep encoding baculovirus in relation to two other baculoviruses were used such that the ratio Rep/cap/transgene started from 1:1:1 and continues until 4:1:1 with the intervals of 1. $2x10^6$ cells/ml of the insect cell line *Expres*SF+ (Protein Sciences) were infected with the baculovirus constructs and grown for 72 hours at 28 °C in a shaker incubator. After infection, cells
15 were lysed for 1 hour at 28 °C with 10x lysis buffer (1.5M NaCl, 0,5M Tris-HCl, 1mM $MgCl_2$, 1% Triton x-100, pH=8.5). Next, genomic DNA was digested with 4µl/100ml benzonase (Merck) at 37 °C. Cell debris was pelleted at 1900xg and supernatant was stored at -20°C.

Purification

20 rAAV5 capsids were purified from crude lysates using AVB sepharose (GE healthcare). Resin was washed in 0.2 M HPO_4 pH=7.5 buffer, after which crude lysates were added to the resin at room temperature. Resin was washed again in 0.2 M HPO_4 pH=7.5 buffer. Next, bound virus was eluted from the resin by the addition of 0.2M Glycine pH=2.5. pH of eluted virus was adjusted by the addition of 0.5M Tris-HCl
25 pH=8.5. In order to minimize infectivity loss due to the exposure to low pH conditions, elution time was kept as short as possible.

SDS-PAGE gel electrophoresis

To check if AAV5 capsids were properly purified, a Nupage gel (Invitrogen) was
30 run and proteins were visualized with simply blue safe stain (coomassie blue, Invitrogen).

Western Blot analysis

To asses the Rep expression during the experiment, samples were taken on day 1, 2 and 3. To each sample 300µl 10x lysis buffer (1.5M NaCl, 0,5M Tris-HCl, 1mM $MgCl_2$, 1% Triton x-100, pH=8.5) was added and the samples were incubated at 4 °C for 30 minutes. To degrade DNA 0.5µl Benzonase (Merck, cat no 1.01697.0001) was
5 added and samples were incubated for 15min at 37°C. The cell debris was removed by centrifugation at 1900xg for 15minutes. Next, 90 µL of the supernatant was mixed with 30 µL 4x NuPage LDS buffer containing DTT after which samples were boiled for 5 minutes at 95 °C. Western blots were performed according to RD-SOP-PRO-003-version 1 and RD-SOP-PRO-004-version 1. First antibody incubation was done with
10 anti-Rep 303.9 antibody (dilution 1:500; Progen, cat. no. #65169). Polyclonal rabbit anti-mouse IgG-HRP (DAKO, cat no P0260) was used as the secondary antibody in a 1:1000 dilution. To visualize Rep bands, HRP was detected using the Light plus substrate solution (Roche, cat no 12015196001), after which the luminescent signal was detected on the ImageQuant (GE Healthcare).

15 DNA isolation, alkaline gel electrophoresis

To investigate the DNA present in purified AAV5 capsids alkaline gel eelctrophotresis was performed. To isolate DNA from the purified capsids, DNA was released by diluting in 2x Proteinase-k buffer (10mM Tris-HCl, 100mM NaCl, 10mM EDTA, 0.5% SDS, pH=8.0) and incubating for 1 hour in the presence of 2mg/ml
20 Proteinase k (Roche) at 37 °C. DNA was further purified using the Nucleospin DNA extraction kit (Macherey-Nagel). DNA was eluted from spin columns in 0.05M Tris-HCl pH=8.5 buffer and concentrations were measured on the nanodrop system (Thermo Scientific). Eluted DNA was stored at -20 °C.

15µl isolated DNA was run overnight at 15V on an alkaline agarose gel (1%
25 Agarose, 0.05M NaOH, 0.001M EDTA). A 0.05M NaOH, 0.001M EDTA running buffer was used and samples were 5x diluted in an alkaline loading buffer also containing NaOH and EDTA. Bac.VD43, pVD154 and pVD191 DNA digested with BamHI (Invitrogen). After electrophoresis, the gel was stained with SYBR gold (Invitrogen) to show proper separation of DNA.

30 Infectivity assay:

Huh-7 cells were grown in DMEM + GlutaMAXI (Gibco cat no 31966) supplemented with 10% FBS and pen/strep and at 37°C at 5% CO_2. One day prior to

infection cells were seeded in 24-wells plate at a density of $5x10^4$ cells/well and in a final volume of 0.5ml. Purified rAAV5 batches were diluted to a final concentration of $1x10^{11}$ gc/ml with 150mM Glycine/125mM Tris-HCL pH 8.5 solution and 10μl or 100μl was added to the Huh-7 cells to infect with MOIs of $2x10^4$ and $2x10^5$,
5 respectively. To mock infected cells only 10μl or 100μl Glycine/Tris solution was added. Medium of infected Huh-7 cells was harvested 48h and 5 days p.i. and SEAP expression levels were determined using the SEAP reporter gene assay kit (Roche). The assay was performed according the manufacturers protocol (5.4.5) and chemiluminescence is measured on a Spectramax L (Molecular Devices) at a
10 wavelength of 477 nm (A0319 p010-017). The infectivity assay with HEK293 and 84-31 cells was performed as described above with the exception that $2x10^5$ cells/well were seeded, purified rAAV5 batches were diluted to a final concentration of $2x10^{11}$ gc/ml, infections were at a MOI of $1x10^5$ and SEAP activity was measured 24h, 48h and 5 days p.i.
15 The experiment described in the second example was performed as follows: $2x10^4$ Huh7 cells (human hepatoma) were infected with purified rAAV5-Luc virus produced for the ratio scanning experiment. MOI used was $1x10^5$ and Huh7 cells were cultured in DMEM medium (Gibco) supplemented with 10% Fetal bovine serum (Greiner Bio-one). 48 hours and 7 days after infection, luciferase activity was measured
20 in the cells using the One-Glow luciferase detection kit (Promega). Luminescence was measured on a Spectramax L (Molecular Devices) at a wavelength of 540 nm.

Example 1

25 **Introduction**

In the insect cell production system rAAV is generated and encapsidated by two Rep proteins, large Rep78 and small Rep52. These two enzymes have multiple functions and can interact with each other. Rep78 is important in replication and *trs* resolution, whereas Rep52 plays a major role during encapsidation of replicated DNA.
30 Rep52 was shown not to be important for replication reaction, whereas Rep78 although not indispensible seems required for efficient encapsidation. It is known that during replication of AAV genome variety of replication intermediates are formed. These intermediate forms can consist of multiple covalently linked genomes (e.g. replicative

form monomer contains two covalently linked genomes, replicative form dimer –four, etc.). The replicated DNA serves as a substrate for Rep complexes. It first needs to be unwound to a single stranded form and subsequently physically translocated through the pore into the capsid. During or after encapsidation of wild type AAV genome, Rep78 will nick at the terminal resolution site of replication intermediate allowing the encapsidation process to terminate. However, if the encapsidated genome size is half of the maximum encapsidation capacity of wild type AAV the middle ITR can be skipped by Rep78 resulting in encapsidation of 4.7kbp fragment containing two copy of the genome. The present invention discloses that the equilibrium between encapsidation and nicking can be influenced and shifted towards reduction of nicking activity of Rep78 during encapsidation process. This in turns may result in preferential packaging of multimeric genomes.

Rep expression during rAAV production

Here we show that ratio between Rep78 to Rep52 can influence the above mentioned equilibrium and in turn can change the degree of enrichment of vector preparation in monomeric duplex.

A number of different Rep mutants (Fig.1) were used that due to its genetic construction lead to changes in Rep78 and Rep52 expression ratio. Figure 1 shows genetic details of the rep constructs. Rep88 encodes for large Rep78 which contains suboptimal initiation triplet, ACG and Rep 52 that contains strong ATG. The expression of Rep78 is driven from polyhedrin promoter and Rep52 is expressed due to leaky ribosomal scanning. This results in strong expression of Rep78 which increases during production and significantly lower expression of Rep52 (Fig. 2).

Rep183 contains large and small rep proteins under different promoters. Rep78 is cloned under a modified ΔIE1 promoter, which is expressed early following baculovirus infection, whereas Rep52 is expressed from late polyhedrin promoter (Fig. 1). This genetic construction results in early expression of Rep78 which is necessary to replicate the transgene during first production phase and is subsequently silenced during the second production phase when predominantly encapsidation occurs. The expression of Rep52 is strong and most pronounced during second production phase. The ratio Rep78/Rep52 is smaller than 1 and decreases in time (Fig. 2). The other

described mutants due to their genetic organization shows various expression of both large and small Rep proteins as can be deduced from Figure 2.

Formation of monomeric duplex depends on ratio between Rep78 and Rep52

5 rAAV were produced upon baculovirus infection of insect cells with three different baculoviruses, one which carried Rep78/52 cassette encoding two major Replicase enzymes, second Cap cassette encoding viral capsid proteins and third Transgene cassette harboring CMV-seap flanked by inverted terminal repeats (ITR's). The compared rAAV prepared with a number of Rep mutants shows that the skipping 10 of "the middle ITR" is the most efficient when Rep78 to Rep52 ratio is low (i.e. high Rep52 relatively to Rep78). Namely, the construct resulting in generation of the highest enrichment in monomeric duplex is Rep183 (Fig. 3).

rAAV vector enriched in monomeric duplex showed higher potency *in vivo*.

15 Various rAAV vectors prepared with the above mentioned mutants were tested for their potency *in vitro* with three different cell lines as described in materials and methods section (Fig. 4). The activity of reporter gene (seap) was measured as a light emission two and five days post infections with 1e5 virus genome copies. Figure 4 clearly shows that rAAV with the highest genomic content of monomeric duplex 20 transduces the cells most efficiently.

Example 2

25 In order to study the influence of the ratio of three baculoviruses used for rAAV generation on genome encapsidation the titer of the baculoviruses was established (Table 1).

Table 1: Baculovirus titers

Baculovirus stock	titer (iu/mL)	average		
control+	3.81E+08	3.57E+08		1.11
	3.34E+08			
Bac.VD92	5.12E+08	4.27E+08		0.93

	3.42E+08			
Bac.VD154	3.58E+08	4.06E+08		0.98
	4.54E+08			
Bac.VD183	6.8E+08	6.25E+08		0.64
	5.69E+08			
Bac.VD216	2.76E+08	2.9E+08		1.37
	3.03E+08			
Bac.VD263	4.52E+08	3.98E+08		1.00
	3.43E+08			
Bac.pFBDSLR	67475000	89012500		4.47
	1.11E+08			

The obtained titers were used to calculate the required amounts of baculovirus inoculums for infections. The inoculums of Cap encoding baculovirus and transgene encoding baculovirus were kept constant and the Rep encoding baculovirus was increased from 1 to 4 ratio with the increments of 1. Subsequently the insect cells were inoculated with the appropriate amount and type of baculoviruses. The rAAV production was performed as described under materials and methods sections. The samples were taken during three production days for subsequent analysis. At 72h post infections rAAV was harvested and purified as described under materials and methods section and subsequently analyzed.

Expression of Rep proteins during production:

The influence of the elevation of Rep inoculums on its expression was studied. Samples were taken after 24, and 48 h.p.i. and resolved on Nupage gel. The rep expression increases only slightly with increasing inoculums of Rep encoding baculovirus (Figure 5). Similar to example 1 expression driven from Rep183 results in attenuated expression of Rep78 which is the most pronounced at the first phase of production during which the transgene is replicated and continues to decline during second production phase when transgene encapsidation occurs. The increasing expression of Rep52 can be observed over time. The expression of Rep78/52 seems to be reversed when driven from Rep216. Namely, Expression of Rep78 increases over time during the production process, whereas expression of Rep52 declines (Fig.5).

Genome integrity:

Two transgenes were used for generation of rAAV, luciferase (Bac.VD154) and Reporter Gene 2 (Bac.VD263). The size of both of the transgenes is ~3kbp.

Here we demonstrate that by using a certain ratio of Rep78 to Rep52 which is a result of expression of these proteins from Bac.VD183 the preparation of rAAV can be significantly enriched in monomeric duplex genome (Figure 6). Figure 6 shows alkaline electrophoretic resolution of DNA isolated from rAAV produced with three different Rep mutants, FBDSLR, Rep216 and Rep183. The majority of the genome encapsidated by Rep proteins expressed from Bac.VD183 is present in the monomeric duplex form. This appears to be the same for both transgenes tested. In contrast rAAV prepared with FBDSLR shows predominantly the monomer in case of production with luciferase and a mixture of both forms when produced with Reporter Gene 2. rAAV produced with Bac.VD216 contains the mixture of both monomeric genome and monomeric duplex genome (Figure 6).

AAV potency:

In order to test the potency of rAAV generated with various baculoviruses the luciferase reporter assay was performed. Huh7 cells were infected with 1e5 genome copies of rAAV containing luciferase as a reporter gene. The light emission was measured at 2 and 7 d.p.i. Figure 7 clearly shows that the rAAV prepared with Bac.VD183 which contains the highest degree of monomeric duplex genomes is more potent as compared to rAAV generated with Bac.VD216 or FBDSLR which are harbouring predominantly monomeric genomes (Fig. 6). The rAAV prepared with Bac.VD183 is enriched in monomeric duplex as compared to rAAV prepared with Bac.VD216 or FBDSLR which likely is the reason for its higher potency.

Example 3

As shown above the conditions exist under which two covalently linked copies of a vector genome can be encapsidated providing that their size do not exceed the maximum packaging capacity of wild type AAV. Here we show that by reducing the size of the transgene it is possible to encapsidate multiple covalently linked genomes. In other words conditions were found that increased skipping of the *trs*, resulting in a final size of the genome that was packaged which was limited by the physical

maximum capacity of the capsid. This allowed for maximum encapsidation of four consecutive covalently linked copies of the genome for a vector genome of ~1 kbp.

rAAV production

5 rAAV was produced upon baculovirus infection of insect cells with three different baculoviruses, which carried Rep78/52 cassette encoding two major Replicase enzymes, Cap cassette encoding viral capsid proteins and transgene cassette harboring genome of interest i.e. murine U1 flanked by inverted terminal repeats (ITR's). In order to investigate the conditions under which skipping of "the middle ITR" is the most 10 efficient we have compared two Rep variants, namely, Rep88 and Rep183. Figure 1 shows genetic details of both constructs. Rep88 encodes for large transcript on which two reps are encoded; Rep78 which is translated starting from a suboptimal initiation triplet, ACG and Rep52 that contains a strong ATG translation start site. The expression of the large transcript is driven from polyhedrin promoter, the translation of 15 Rep52 is resulting from leaky ribosomal scanning. This results in strong expression of Rep78 which increases during production and significantly lower expression of Rep52.

Rep183 contains large and small rep proteins under different promoters. Rep78 is cloned under modified ΔIE1 promoter, which is express early during baculovirus expression, whereas Rep52 is expressed from late polyhedrin promoter (Fig. 1). This 20 genetic constructions result in early expression of Rep78 which is necessary to replicate the transgene during first production phase and a subsequent decline in expression during second production phase when predominantly encapsidation occurs. The expression of Rep52 is strong and most pronounced during the second production phase. The ratio Rep78/Rep52 is smaller than 1 and decreases in time.

25

Multimeric monomeric duplex

Rep88 and Rep183 baculovirus constructs were used for production of rAAV together with Bac.Cap6 and Bac.mU1 transgene (1065bp). Recombinant vectors were harvested and purified from insect cells constructs using standard procedures. Subsequently DNA 30 was purified from obtained rAAV's and in order to separate it according to its true size alkaline conditions were applied. Figure 8 shows DNA isolated from biological triplicates produce either with Rep88 or with Rep183. rAAV prepared with Rep88 contains predominantly DNA of ~2kbp corresponding to two covalently linked

genomes (Figure 8, panel A). However vector generated with Rep183 besides the tandem genomes contains discrete bands representing three and four covalently linked genomes (Figure 8, panel B). This shows that encapsidation of multimeric genomes observed with Rep183 relates to the ratio between large and small Rep proteins used
5 for rAAV generation. Moreover, the biphasic expression of replicase enzymes which is stronger for Rep78 at the first phase of production and for Rep52 at the second phase contributes to the observed increased encapsidation of multimeric vector genomes.

Claims

1. Use of an insect cell capable of expressing AAV Rep proteins and AAV Cap proteins in the presence of a vector genome comprising at least one AAV ITR, wherein Rep52 and/or Rep40 protein expression and/or activity is increased relative to Rep78 and/or Rep68 protein expression and/or activity in the preparation of monomeric duplex AAV vector.

2. Use according to claim 1, wherein Rep52 and/or Rep40 protein expression is greater than Rep78 and/or Rep60 expression.

3. Use according to claim 1 or 2, wherein the nicking activity of Rep78 and/or Rep68 is reduced relative to the helicase/encapsidation activity of Rep52 and/or Rep40 in the preparation of monomeric duplex AAV vector.

4. Use according to claim 3, wherein the nicking activity of Rep78 and/or Rep68 is less than that of the helicase/encapsidation activity of Rep52 and/or Rep40.

5. Use according to any one of the preceding claims, wherein the vector genome comprises two AAV ITRs, each comprising functional terminal resolution sites.

6. Use according to any one of the preceding claims, wherein at least about 70% of the AAV vector thus produced comprises AAV genome in the form of a monomeric duplex.

7. Use according to any one of the preceding claims, wherein the insect cells are infected with at least a first recombinant baculovirus harboring an AAV vector nucleotide sequence and a second recombinant baculovirus harboring a Rep78 and/or Rep 68 encoding nucleotide sequence and a Rep52 and/or Rep 40 encoding nucleotide sequence and wherein the multiplicity of infection (m.o.i.) of the recombinant baculovirus harboring the Rep encoding sequences is the higher.

8. Use according to any one of the preceding claims, wherein a biphasic level of expression of Rep78/68 and Rep52/40 proteins occurs.

9. Use according to claim 8, wherein the level of Rep78 and/or Rep68 protein
5 expression is increased relative to Rep52 and/or Rep40 protein expression in the first phase and the level of Rep52 and/or Rep40 protein expression is increased relative to Rep78 and/or Rep40 protein expression in the second phase.

10. Use according to any one of the preceding claims, wherein Rep52 and/or Rep40
10 protein expression is increased relative to Rep78 and/or Rep68 protein expression during at least part of the encapsidation phase.

11. Use according to any one of the preceding claims, wherein the insect cell expresses: Rep78 and Rep 52 proteins, and no Rep68 or Rep 40 proteins; or Rep68 and
15 Rep 40 proteins and no Rep78 or Rep 52 proteins.

12. Use according to any one of the preceding claims, wherein the vector genome is smaller in length than a wild type AAV genome.

20 13. Use according to claim 12, wherein the resulting AAV comprises a partial monomeric duplex genome.

14. Use according to any one of the preceding claims, wherein the vector genome is less than about 2.5 kb and results in the encapsidation of a concatamer of monomeric
25 duplex genomes.

15. Use according to any one of the preceding claims, wherein a first promoter active in insect cells is used to drive the expression of Rep78 and/or Rep68 proteins and a second promoter active in insect cells is used to drive the expression of Rep52 and/or
30 Rep40 proteins, the activity of the first promoter being relatively weaker as compared with the activity of second promoter in said insect cells.

16. Use according to any one of the preceding claims, wherein nucleotide sequences encoding the Rep78 and/or Rep68 proteins and the Rep52 and/or Rep40 proteins are present on separate expression vectors.

5 17. Use according to any one of claims 1 to 15, wherein the Rep78 (and/or Rep68) protein and the Rep52 (and/or Rep40) protein are encoded by and expressed from a single open reading frame.

18. Use according to any one of claims 1 to 15, wherein the Rep52 and/or Rep40
10 protein is expressed from an internal promoter located in an intron sequence present in the Rep78 and/or Rep68 encoding sequence in insect cells.

19. Use of an insect cell capable of expressing AAV Rep proteins and AAV Cap proteins in the presence of a vector genome comprising at least one AAV ITR, wherein
15 the Rep proteins are expressed from a nucleotide sequence as set out in any one of SEQ ID NOs: 1 to 7 in the preparation of monomeric duplex AAV vector.

20. Use of an insect cell capable of expressing AAV Rep proteins and AAV Cap proteins in the presence of a vector genome comprising at least one AAV ITR, wherein
20 the Rep proteins are expressed from a construct as set out in Fig. 1 as Bac.VD183 in the preparation of monomeric duplex AAV vector.

21. Use of a Rep encoding sequence according to any one of SEQ ID NOs: 1 to 7 in the preparation of monomeric duplex AAV vector in insect cells.

25

22. Use of a construct as set out in Fig. 1 as Bac.VD183 in the preparation of monomeric duplex AAV vector in insect cells.

23. A method for the preparation of monomeric duplex AAV vector, said method
30 comprising expressing in insect cells AAV Rep proteins and AAV Cap proteins in the presence of a vector genome comprising at least one AAV ITR, wherein Rep52 and/or Rep40 protein expression is increased relative to Rep78 and/or Rep68 protein expression.

24. A method according to claim 23, wherein Rep52 and/or Rep40 protein expression is greater than Rep78 and/or Rep60 expression.

5 25. A method for the preparation of monomeric duplex AAV vector, said method comprising expressing in insect cells AAV Rep proteins and AAV Cap proteins in the presence of a vector genome construct flanked by at least one AAV ITR, wherein the nicking activity of Rep78 and/or Rep 60 is reduced relative to the helicase/encapsidation activity of Rep52 and/or Rep 40.

10

26. A method according to claim 25, wherein the nicking activity of Rep78 and/or Rep68 is less than that of the helicase/encapsidation activity of Rep52 and/or Rep40.

27. A method according to any one of claims 23 to 26, wherein the vector genome
15 comprises two AAV ITRs, each comprising functional terminal resolution sites.

28. A method according to any one of claims 23 to 27, wherein at least about 70% of the AAV vector thus produced comprises AAV genome in the form of a monomeric duplex.

20

29. A method according to any one of claims 23 to 28, wherein the insect cells are infected with at least a first recombinant baculovirus harboring an AAV vector nucleotide sequence and a second recombinant baculovirus harboring a Rep78 and/or Rep 68 encoding nucleotide sequence and a Rep52 and/or Rep 40 encoding nucleotide
25 sequence and wherein the multiplicity of infection (m.o.i.) of the recombinant baculovirus harboring the Rep encoding sequences is the higher.

30. A method according to any one of claims 23 to 29, wherein a biphasic level of expression of Rep78/68 and Rep52/40 proteins occurs.

30

31. A method according to claim 30, wherein the level of Rep78 and/or Rep68 protein expression is increased relative to Rep52 and/or Rep40 protein expression in

the first phase and the level of Rep52 and/or Rep40 protein expression is increased relative to Rep78 and/or Rep40 protein expression in the second phase.

32. A method according to any one of claims 23 to 31, wherein Rep52 and/or Rep40 protein expression is increased relative to Rep78 and/or Rep68 protein expression during at least part of the encapsidation phase.

33. A method according to any one of claims 23 to 32, wherein the insect cell expresses: Rep78 and Rep 52 proteins, and no Rep68 or Rep 40 proteins; or Rep68 and Rep 40 proteins and no Rep78 or Rep 52 proteins.

34. A method according to any one of claims 23 to 33, wherein the vector genome is smaller in length than a wild type AAV genome.

35. A method according to claim 34, wherein the resulting AAV comprises a partial monomeric duplex genome.

36. A method according to any one of claims 23 to 35, wherein the vector genome is less than about 2.5 kb and results in the encapsidation of a concatamer of monomeric duplex genomes.

37. A method according to any one of claims 23 to 36, wherein a first promoter active in insect cells is used to drive the expression of Rep78 and/or Rep68 proteins and a second promoter active in insect cells is used to drive the expression of Rep52 and/or Rep40 proteins, the activity of the first promoter being relatively weaker as compared with the activity of second promoter in said insect cells.

38. A method according to any one of claims 23 to 37, wherein nucleotide sequences encoding the Rep78 and/or Rep68 proteins and the Rep52 and/or Rep40 proteins are present on separate expression vectors.

39. A method according to any one of claims 23 to 37, wherein the Rep78 (and/or Rep68) protein and the Rep52 (and/or Rep40) protein are encoded by and expressed from a single open reading frame.

5 40. A method according to any one of claims 23 to 37, wherein the Rep52 and/or Rep40 protein is expressed from an internal promoter located in an intron sequence present in the Rep78 and/or Rep68 encoding sequence in insect cells.

41. A method for the preparation of monomeric duplex AAV vector, said method
10 comprising expressing in insect cells AAV Rep proteins and AAV Cap proteins in the presence of a vector genome comprising at least one AAV ITR, wherein said Rep proteins are expressed from a sequence as set out in any one of SEQ ID NOs: 1 to 7.

42. A method for the preparation of monomeric duplex AAV vector, said method
15 comprising expressing in insect cells AAV Rep proteins and AAV Cap proteins in the presence of a vector genome comprising at least one AAV ITR, wherein said Rep proteins are expressed from a construct as set out in Fig. 1 as Bac.VD183.

43. A monomeric duplex AAV vector produced by use according to any one of
20 claims 1 to 22 or a method according to any one of claims 23 to 42.

44. A composition comprising AAV vector of which at least about 70% is in the form of monomeric duplex AAV vector.

25 45. A pharmaceutical composition comprising a monomeric duplex AAV vector according to claim 43 or a composition according to claim 44 and a pharmaceutically acceptable carrier or diluent.

46. A method for delivering a nucleotide sequence to a cell, which method comprises
30 contacting a monomeric duplex AAV vector according to claim 43, a composition according to claim 44 or a pharmaceutical composition according to claim 45 under conditions such that the monomeric duplex AAV enters the cell.

47. A method for administering a nucleotide sequence to a subject, which method comprises adminstering to the said subject a monomeric duplex AAV vector according to claim 43, a composition according to claim 44 or a pharmaceutical composition according to claim 45.

5

48. Use of a monomeric duplex AAV vector according to claim 43, a composition according to claim 44 in the manufacture of a medicament for use in the administration of a nucleotide to a subject.

10

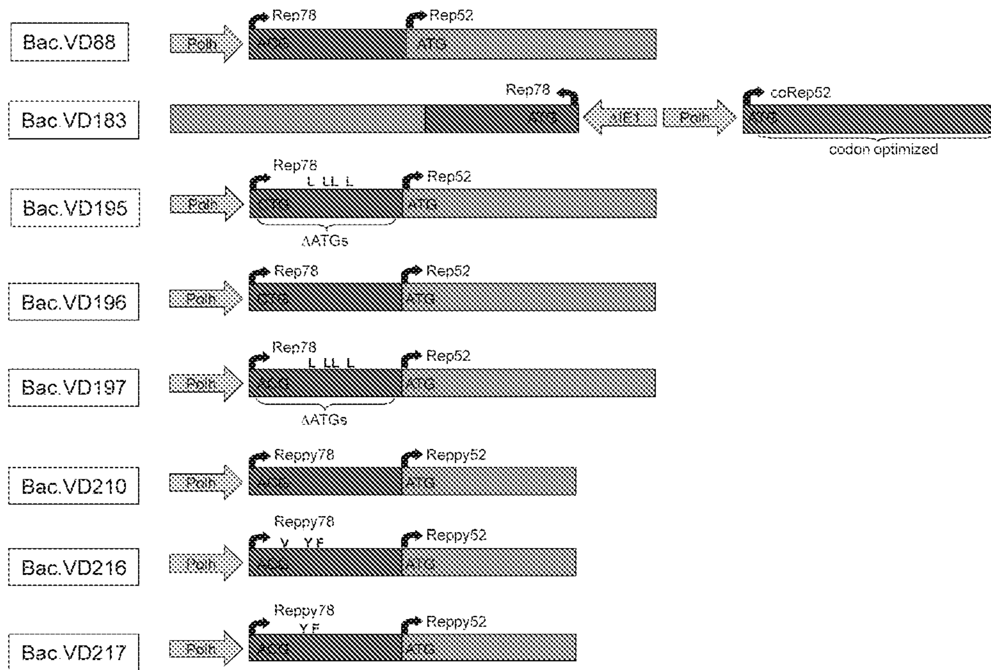

Fig. 1

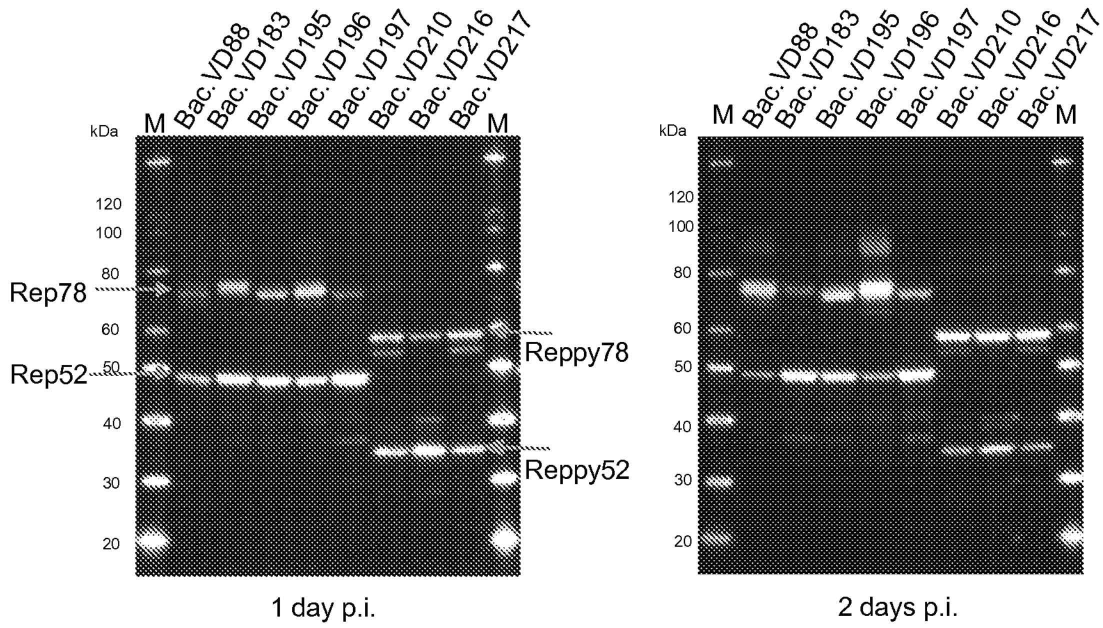

Fig. 2

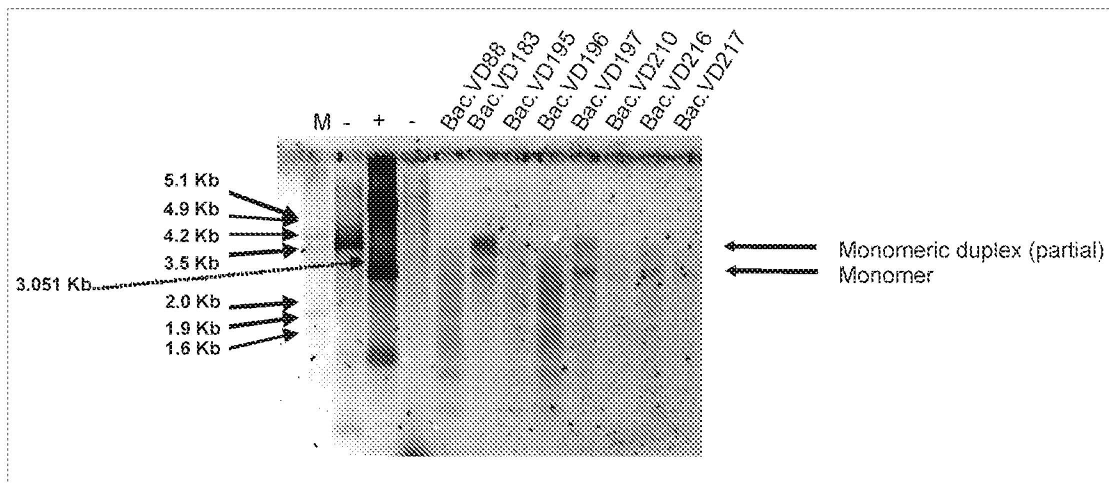

Fig. 3

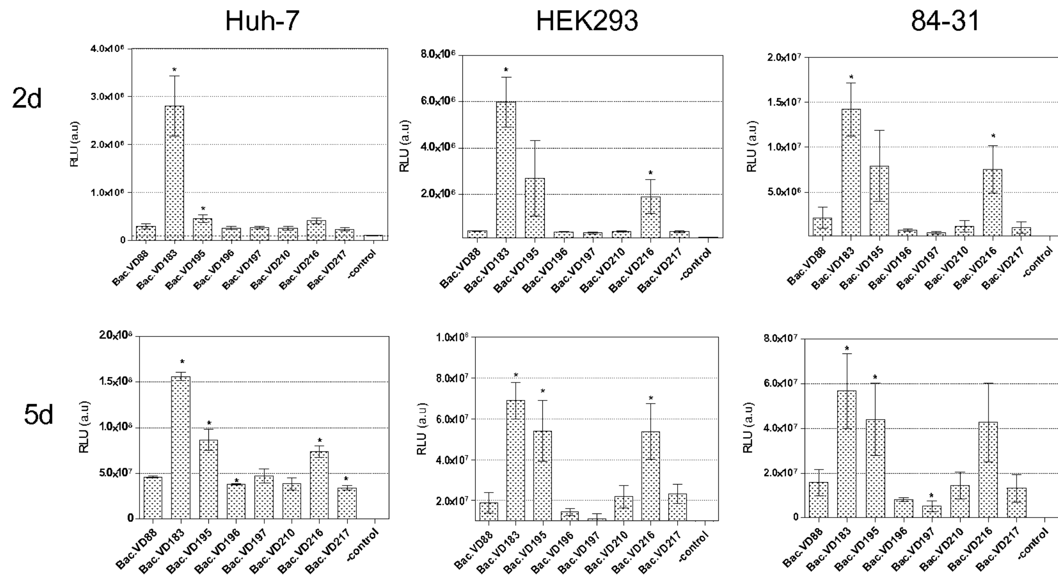

Fig. 4

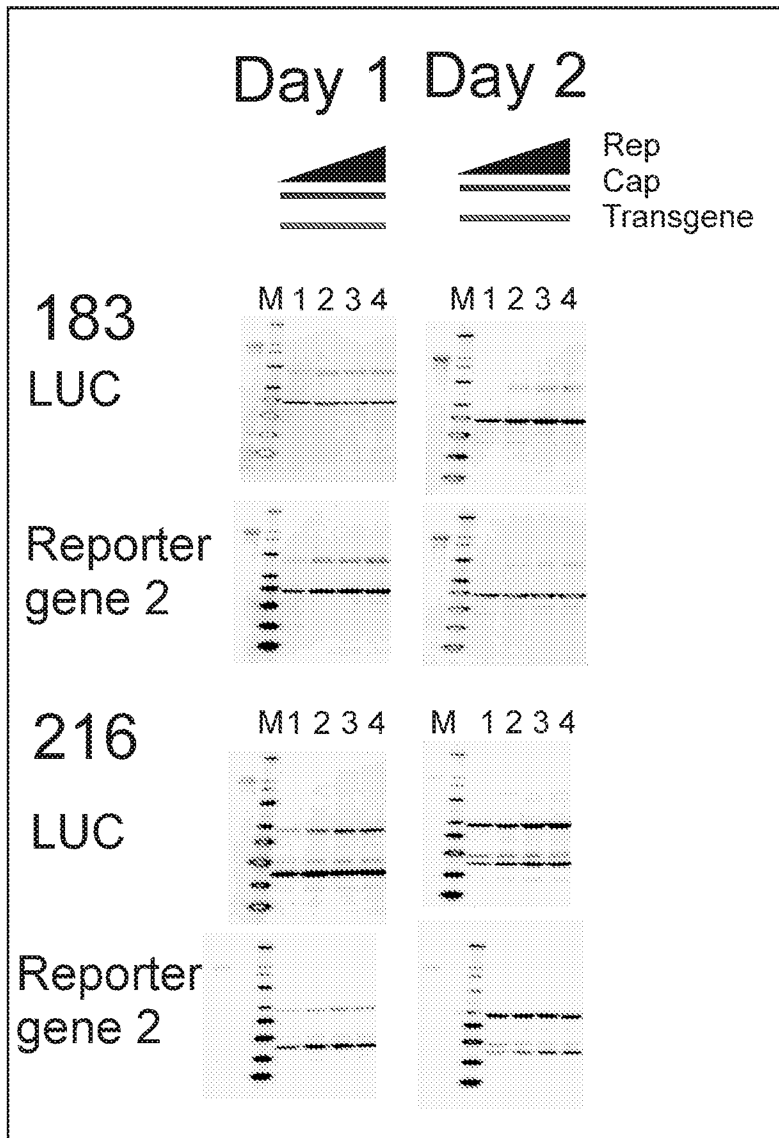

Fig. 5

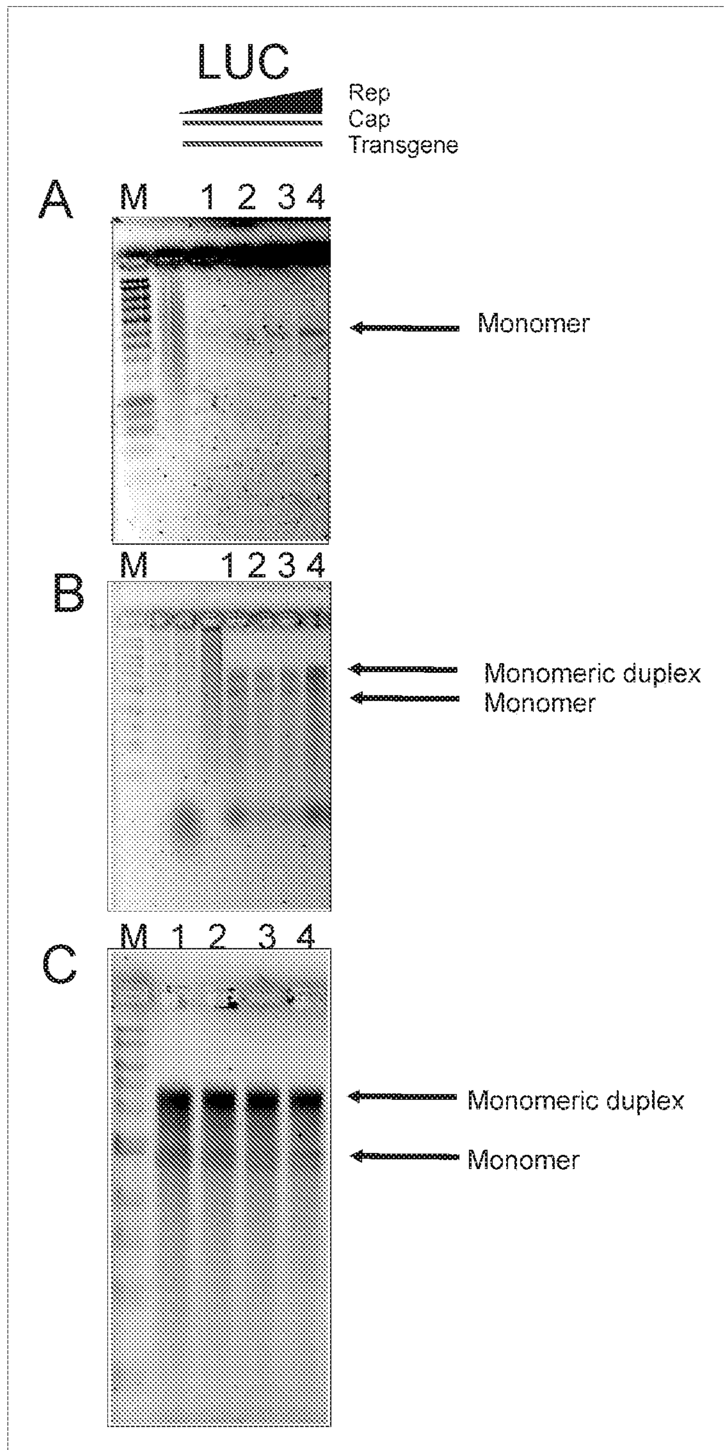

Fig. 6

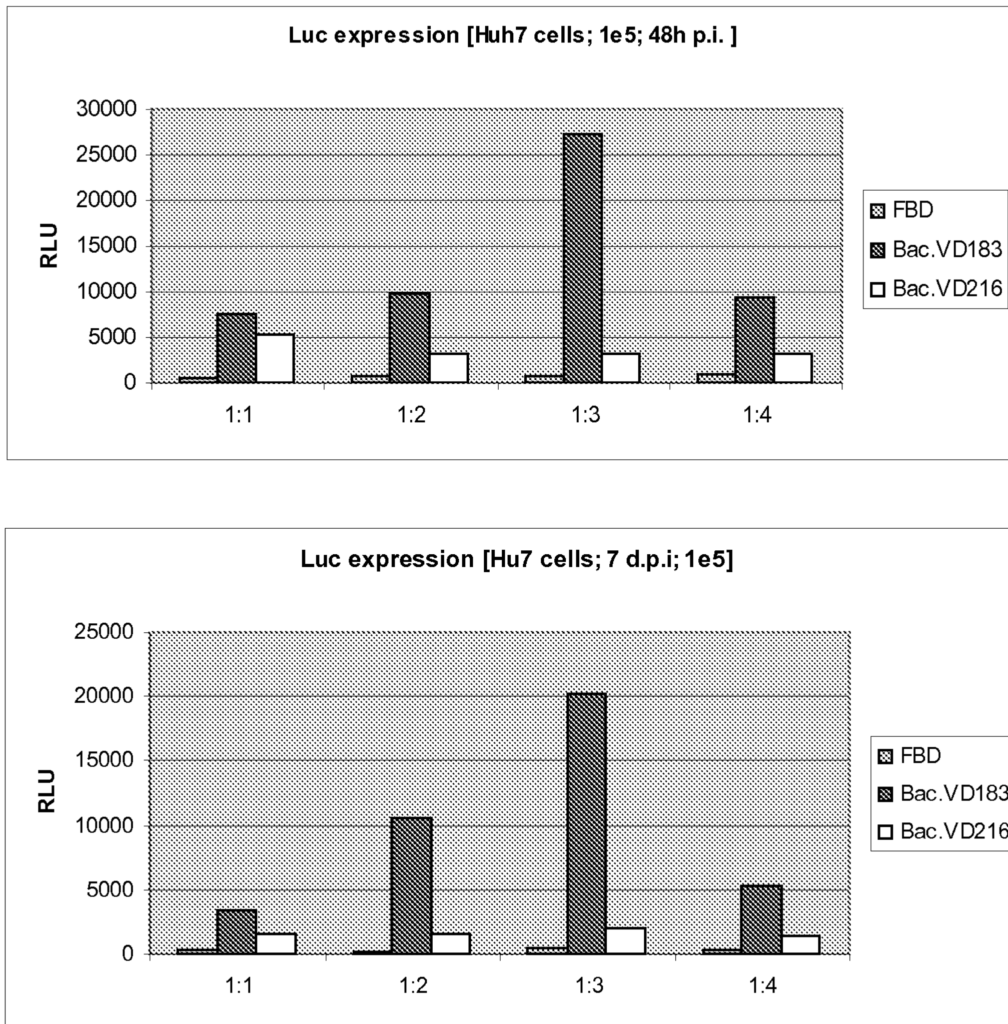

Fig. 7

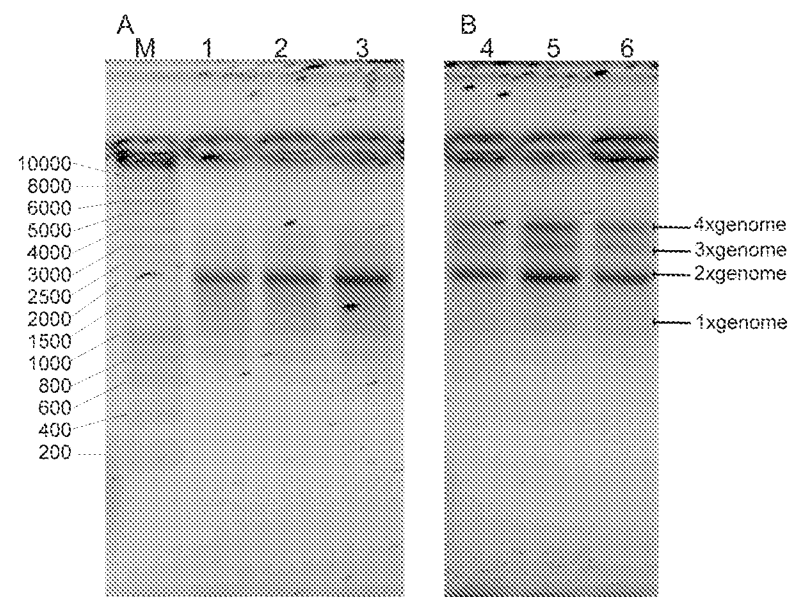

Fig. 8

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2011/050221

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/015 C12N15/864 C12N7/02
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N

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

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

EPO-Internal, BIOSIS, MEDLINE, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/075861 A2 (CHILDRENS HOSPITAL INC [US]; CLARK KELLY REED [US]; JOHNSON PHILIP R J) 10 September 2004 (2004-09-10) the whole document -----	1,2,5-7, 11-18, 23,24, 27-29, 33-40, 43-48
X	WO 2007/148971 A2 (AMSTERDAM MOLECULAR THERAPEUTI [NL]; HERMENS WILHELMUS THEODORUS JO [N) 27 December 2007 (2007-12-27) cited in the application the whole document -----	1,2,5-7, 11-18, 23,24, 27-29, 33-40, 43-48

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 August 2011	30/08/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Wimmer, Georg

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2011/050221

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 7 115 391 B1 (CHEN HAIFENG [US] ET AL) 3 October 2006 (2006-10-03) the whole document -----	1,2,5-7, 11-18, 23,24, 27-29, 33-40, 43-48
X	US 5 622 856 A (NATSOULIS GEORGES [US]) 22 April 1997 (1997-04-22) the whole document -----	1,2,5-7, 11-18, 23,24, 27-29, 33-40, 43-48
X	US 6 475 769 B1 (WILSON JAMES M [US] ET AL) 5 November 2002 (2002-11-05) the whole document -----	1,2,5-7, 11-18, 23,24, 27-29, 33-40, 43-48
X	US 6 482 634 B1 (WILSON JAMES M [US] ET AL) 19 November 2002 (2002-11-19) the whole document -----	1,2,5-7, 11-18, 23,24, 27-29, 33-40, 43-48
X	WO 2009/014445 A2 (AMSTERDAM MOLECULAR THERAPEUTI [NL]; BAKKER ANDREW CHRISTIAN [NL]; HER) 29 January 2009 (2009-01-29) the whole document -----	1,2,5-7, 11-16, 20, 22-24, 27-29, 33-38, 42-48
X	URABE MASASHI ET AL: "Scalable generation of high-titer recombinant adeno-associated virus type 5 in insect cells", JOURNAL OF VIROLOGY, THE AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 80, no. 4, 1 February 2006 (2006-02-01), pages 1874-1885, XP002429140, ISSN: 0022-538X, DOI: DOI:10.1128/JVI.80.4.1874-1885.2006 the whole document -----	1,2,5-7, 11-16, 20, 22-24, 27-29, 33-38, 42-48

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL2011/050221

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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

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

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

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

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

see additional sheet

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

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

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

   2, 15, 20, 22-24, 37, 42(completely); 1, 5-7, 11-14, 16-18, 27-29, 33-36 38-40, 43-48(partially)

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

Remark on Protest

☒ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/NL2011/050221

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 2004075861	A2	10-09-2004	NONE			
WO 2007148971	A2	27-12-2007	AU	2007261806	A1	27-12-2007
			CA	2655957	A1	27-12-2007
			EP	2035564	A2	18-03-2009
			JP	2009540823	A	26-11-2009
			KR	20090037401	A	15-04-2009
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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 2, 15, 23, 24, 37(completely); 1, 5-7, 11-14, 16-18, 27-29, 33-36, 38-40, 43-48(partially)

   Method for the preparation of a monomeric duplex AAV vector, comprising expressing in insect cells AAV Rep and AAV Cap proteins in the presence of a vector genome comprising at least one AAV ITR, and corresponding uses of insect cells, wherein Rep52 and/or Rep40 protein expression is increased relative to Rep78 and/or Rep68 protein expression.

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2. claims: 3, 4, 25, 26(completely); 1, 5-7, 11-14, 16-18, 27-29, 33-36, 38-40, 43-48(partially)

   Method for the preparation of a monomeric duplex AAV vector, comprising expressing in insect cells AAV Rep and AAV Cap proteins in the presence of a vector genome comprising at least one AAV ITR, and corresponding uses of insect cells, wherein the nicking activity of Rep78 and/or Rep68 is reduced relative to the helicase/encapsidation activity of Rep52 and/or Rep40.

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3. claims: 8-10, 30-32(completely); 11-14, 16-18, 27-29, 33-36, 38-40, 43-48(partially)

   Method for the preparation of a monomeric duplex AAV vector, comprising expressing in insect cells AAV Rep and AAV Cap proteins in the presence of a vector genome comprising at least one AAV ITR, and corresponding uses of insect cells, wherein a biphasic level of expression of Rep78/68 and Rep52/40 proteins occurs.

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4. claims: 19, 21, 41, 43-48(all partially)

   Method for the preparation of a monomeric duplex AAV vector, comprising expressing in insect cells AAV Rep and AAV Cap proteins in the presence of a vector genome comprising at least one AAV ITR, and corresponding uses of insect cells, wherein said Rep proteins are expressed from the Sequence of SEQ ID NO. 1.

5-10. claims: 19, 21, 41, 43-48(all partially)

   As invention 4, but wherein the Rep proteins are expressed from sequences selected from SEQ ID NOs. 2-7.

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11. claims: 20, 22, 42(completely); 43-48(partially)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

As Invention 4, but wherein the Rep proteins are expressed from a construct as set out in Fig. 1 as "Bac.VD183".