Abstract: Compositions and methods are disclosed for producing adeno-associated virus (AAV) in insect cells in vitro. Recombinant baculovirus vectors include an AAV Capsid gene expression cassette (Cap), an AAV Rep gene expression cassette (Rep), and a baculovirus homologous region (hr) located up to about 4 kb from a start codon in an AAV expression cassette. Production levels of baculovirus and AAV in insect cells harboring recombinant baculovirus comprising a Cap, a Rep, and an hr are higher compared to controls comprising a Cap and a Rep but no hr. Furthermore, levels of baculovirus and AAV production in insect cells infected with recombinant baculovirus comprising a Cap, a Rep, and an hr are comparatively stable over serial passages of cells, whereas levels of baculovirus and AAV production decline over serial passages of insect cells comprising recombinant baculovirus comprising a Cap and a Rep, but no hr.

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AAV Production in Insect Cells, Methods and Compositions Therefor

Reference to Prior Application

This application claims benefit of and priority to US Provisional Application 62/325,817 filed April 21, 2016. Application 62/325,817 is hereby incorporated by reference in its entirety.

Incorporation by Reference of Sequence Listing

The Sequence Listing, which is a part of the present disclosure, includes a computer readable form and written sequence listing comprising nucleotide and/or amino acid sequences. The sequence listing information recorded in computer readable form is identical to the written sequence listing. The subject matter of the Sequence Listing is incorporated herein by reference in its entirety.

Introduction

With the approval of the first adeno-associated virus (AAV)-mediated gene therapy drug, the demand for large-scale AAV vector manufacturing technology is ever increasing (Yla-Herttuala, S., Mol. Ther. 20, 1831-1832, 2012). Currently there are several technologies for producing AAV. The traditional method utilizes transfection of HEK293 cells or other mammalian cell lines with triple or double plasmids. This method has low yields of AAV and is difficult to scale up due to its requirement for adherent cells (Xiao, X., Li, J., & Samulski, R. J., J. Virol. 72, 2224-2232, 1998). Another method for producing AAV utilizes Herpes Simplex virus (HSV) to infect mammalian cells. This method is hampered by difficulties in generating sufficient HSV seed stocks, and also has low AAV productivity (Booth, M.J., et al., Gene Ther. 11, 829-837, 2004).

Baculovirus-based methods for AAV vector production in insect cells have significantly increased AAV production yields compared to other systems (Urabe, M., et al., Human Gene Therapy 13, 1935-1943, 2001; Chen, H., Mol. Ther. 16, 924-930, 2008; Chen, H., Molecular Therapy-Nucleic Acids 1, e57, 2012; US Patent 8,945,918 to Chen). However, recombinant baculovirus can be unstable over multiple passages, leading to a decline in AAV production. There is thus a great need for an AAV production system that can maintain production of AAV vectors at a high yield even after multiple passages.
The baculovirus Autographa califomica multiple nuclear polyhedrosis virus (AcMNPV) genome includes 5 "homologous regions" (hrs) designated hrl-hr5 (Cochran, M.A., and Faulkner, P.J., Virol. 45, 961-970, 1983; Guarino, L.A. and Summers, M.D. J. J. Virol. 60, 214-223, 1986). These 5 regions can function as enhancers (Guarino, L.A., et al., J. Virol. 60, 224-229, 1986). Sequences of hrl-hr5 have been reported (Guarino, L.A. and Summers, M.D., J. Virol. 60, 214-223, 1986; Guarino, L.A., et al., J. Virol. 60, 224-229, 1986) and are set forth herein. An hr can be from about 400 base pairs up to about 1,000 base pairs in length. Examples of sequences of AcMNPV hrs are as follows.

hn: ATCGATGATT GACCCCAACA AAGATTTAT AATTAATCAT AATCACGAC
AAACAAAGT CATAAAACA AATAAACAGG TTGTCGATAA AACATTCAA
AATGACACAG CAACATACAA TTCTTGATA ATAAAATTT AAAATGACATC
ATATTTGAGA AATAAAATGT CATATTATTC TCGATTTGTT TTTACAGTA
GAATTCCTAC CGTAAAGCCG CTTGAGTTTTTTT GAAAAACAAA TGATGATCTT
TGATAATGACATCATCCCC TGAAGGTAATTTACCGTAG AATTCATCC
GTAAGCGAG TTCAGTTTTT AAAACAAATG GATGATACCT AAACACGTTA
ATAATCTTCT GATATCAGCT TAGACTCAA GTATGAGCC GTGTCGAA
CATGAGATAA GTTTTAGACA TCATCAGCTG ATCGTGGATT CAAATGAGAA
TTCTACTCGT AAAGCCAGTT CGTTATGAG CCGTGTCGAA AACATGACAT
CAGCTTATGTA CTCATACTTGG ATTTGAGATT ACGCGATGAA TTCTACTCGT
AAAGCGAGTT CGTATTAGAG CGATGGTCGAA AACATGACAT CAGCTTATGA
GTCATAATTA ATCGTGGATT ACAATGAGAA TTCTACTCGT AAAGCGAGTT
GAAGGATCT AATTAAGTGTA TTGATGAGA TAAGATGAA AAGCGTGGAA
AAATTTGCG CCAGCTTGGCA CAACTATTCA CAATGCAGCC AAGTTTACAA
AGATTCTAT CTGATATGG ATTAACACCC TTTGCGGCGCC GATTTTTTGG
CGTACGTCG TAGCGAAGAA GATGGTTGGA CCGCAGAACA GATAGTAAAA
CAAACCCCTA GTATTTGAGC AATAATCGAT (SEQ ID NO: 1)

hr2: TGAGCAAAAC ACAACCGGCA AATTCCTCGGC GGGCGTTTGG GAAATGCGGAA
TAATTGCCCAT ATGTAATAGA TGTCATCGGT TCTAATCTGC TTTCAGAGTA
GAATTCCTAC TGTAACCAACAA TAATGACAGA TGATGTATTG TGGTTTTTCAA
AACTGAACTC AAGAAATGAT GTCATTTG GTTTCAAAACT GAATGGCTTT
TACGATAGA AATTTACTTG TAAACACAA TCGAGAGATG ATGTCATATT
TTGCACACGG CTCAAATTAA ACTGCGTTGA CGAGTAATAT TCTACTTGTAA
ACGCTATGATC AAGGGATGAT GTCATTGGAT GAGTCATTTG TTTTCAAAAA
CTAAACTCGG TTTACGAGTA GAATTCTACT TGAAAAACAC AATCAAAGGGGAT
TATGTCATT ATACAAATGA TGTCATTGGT TTTTCAAAAAC TAAAACGTGCT
TTACGGGTAG AATTCTACTT GTAAAAACAG AACTCGAGGG ATGATGTCAT
CTTTTACTCG ATGATTATAAA ACGITTTTAT GATGACTCIA TTTTTTTTTC
AAAATCAAC TCGCTTTACG AGTAGAATTC TACTTGTAAAC GCACGATCAA
GGGATGATGT CATTATTTTG TGCAAAGCTC GATGTCATCT TTTGACACAG
ATTATAAAC CAATCCCAAAT AATGACTCAT TGTTTTTCAA AACTGAACCTC
GCTTTACAG TGAATTTCTA TTGGTAACAC ACAATCAAAGG GATGATGCTA
TTTTCAAAAT GATGACTATT GTTTTTCAA ACTAAAATCG CTTTACGAGT
AAATCTCAC TTGTAAAACA CAATCAAGGG ATGATGTCAT TTAAAAATAT
ATCATTGTTTT TTCTAAAACT AACTCGCATT TACGAGTATA ATTCCTACGTG
TTAAAAACAA TCAAGGGATG ATGTCATTCT CAATAAAAAA TAATTTTTTA
AATAAAAACTG TTTTTATTTG TCAATACAC ATTGATTTCAC (SEQ ID NO: 2)

hr3: ACGCGTAGAA TTCTACTTGT AAAGCAAGTT AAAATAAGCC GTGTGCAAAA
ATGACATCAG ACAAATGACA TCATCTACCT ATCATGATCGA TGTTAATAAT
CATGTTTTAA AATGACATCA GCTTTATGACT AATAATGGAT CGTGCCTTAC
AAGTGAATTT CCTACTCGTAA AGCGAGTTTA GITTGAAAAA CAAATGAGTC
ATCATAAAC ATGTGTAATAA TCGTGATATA AGGATGACAT CATCCACTAA
TCGTCGTCAA AAGATGAAAT TCTACTCGTAA AAGCGAGTTTT
AACAATGAC ATCATTTCTT GATGGTGTTT TACAGTATA ATTCCTACGTG
TAAAGATGTG TCGTGTAAAA AAACAAATGA CATCATTTTA CAGATGACAT
CATTCTCGA TTATGTTTAA CAAGTGAATTT TCTACTCGTAA AAGCGAGTTT
AGTTTTAAAA AACAATGAC ATCATTCTTT GATGGTGTTT TACAGTATA
ATTCTACTCG TAAAGCGAGTT TAGTTTTTGA AAAAAAATG ACATCATCTC
TTGATTATGT TTTACAAGTTA GAATCTACGTGA AAGAGCAGGA GTTTAGTTTTT
GAAAAAACAAG TACATCATC CCTTGATCAT GCGTTACAAG TAGTAATCTTTA
CTGTAAGACG GAGTGGATTT TTGATTACAAGA TATT (SEQ ID NO: 3)

hr4 left: ATGCATATATTT TGTGTACAAA AATATGACTC ATTAATCGAT
CGTGCCTTTAC AAGTGAATTCT CTACTGGTTAA AGCAAGTTTCG GTGTGAGGCC
GTGTGCAAAA CATGACATCA TAACATCATA TGTTTTAATAT CATGTGCAA
AATAGCAGTAC ATCCGAGCAT GTGTGTTTAC AAGTAGAATT CTACTCGTAA
AGCGAGTTTA AAAAAATGT GACGTCAATTG AAACACGTTG TAAATTTTTTT
TACAATATTG AAGTGAACCA TTATGACTTCA AATATTTTTT GTGGAATGTTG

3
ATACGTTTGGC AAGACAATTTG ATTACAGATA AATGTAGTGC TCTAATCGAA
AGATGCAGGAT CTGGTGGCGGG CAAAACATTIT AGAGATTTAGT AGAGAAAGGC
CAGACACAAAG TATTTTGAAG TGCCAACCTCA AAAAAACTAT GAATAACATTA
AAAAATATT TTATACGAACA AAATAATATGG AGCATTCAGAT AGATTATAAAA
GATTAAAAAC GACGCACTCCT ATGGGATAGTT TTTAAATTCC CTTAAAACAA
GAGCACAACCC TACTTTCCCAT CGTACTAAAG AGATCATCAGA GTGGGCGCAT
AAACGTITAA ACAAAAAATTA CCCCGATTAA AAGAGTTTCTC CGCGCAATGAC
TCAGCATTACAAATGAATG TTTGGAAAAT CTAGA (SEQ ID NO: 4)

hr4 right: AACTGGCCTTT ACGAGTAGAA TTCTACTTGT AAAACACAAAT
CAAGAAATGAG TCTGGCGATT ATAAACATGTT TTAAACATGG
TACATTGAAC TTAATTTTGA CAAAGTTGAA AACTAGATT ATGTAATGACT
CATTGGTTTG TGCAAGTTGAG TAAACGTGAT TAATATATGA CTGATATTTT
TGTCGAAAAA TGGTGTCACTC ATCAAAAATCC GCTTTACGAG TAGAATTCTA
CTGTTAAAAAC ACAATCAGGAG GATAGTAGTA TTTGAGAAT GATGTCATT
GTGTTTTCGA AACCAAAACTC GCTTTACGAG TAGAATTCTA CTTGTTAAAAC
ACAATCGAGG GATGAGTAGCA TTTGTAGAAT GATGTCATCG TACAAAACCTG
CTTACGAGT AGAATTCTTAG AAAACACAC (SEQ ID NO: 5)

hr5: TTGAAAATTT ATTCGCTTAAT ATTATTTTTG TCGTTTCGTT GTACTTTATT
AATTTGGATG ATGTCCATTGTT GTTTTTTAAA TGAACCTGGC TTTACGAGTA
GAATTCTACG CGTAAACAC AATCAAGTAT GAGTCATAAG CTGAATTCAT
GTTTTCACA CGCTCTATAA CCGACTGGGC TTTACGAGTA GAATTCTACTT
TGTAACGCAC GACTCGAGTTG ATGATGGGCA TTTGTTTTTC AAATCGAGAT
GATGTCATGT TTTCACACCG GGTCTCATAA ATGCTTTACG AGTAGAATT
TCATGTAAC GCACGATCAGA TTGATGAGTC ATTTTTTTG CAATATGATA
TCATAACAATA TGACTCATTGTT GTTTTTTAAA ACGAACTTGG ATTTACCGGT
AGAATTCTAC TCGTAACAGCA CAATCAAAAA GATGATGTC TTTGTTTTNC
AAAACCTGAAC TCTGGCCTTT ACGAGTAGAA TTCTACGGTT AAAACACAAAT
CAAGAAATGAG TGTAATTTGT TATAAAAATA AAAGGTATAG TCATGTTTTG
CAGATCCCTC ATAAACATTAC TCGTTTACG GTGAGAATTTC TACGGCTAAA
ACATGATTGAA TAAATACATA ATTCATTTCG TAAACCAATCGA ACGGACGTAA
AACTGGTATAA TGAATTTTGA TAAATATTAA TATGACATGG ATCCAACAAAA
TAAAATATATA ATAGAGCAAG TCGAC (SEQ ID NO: 6)

In addition, sequence diversity amongst hrs in insect viruses has been reported. In addition, sequence diversity amongst hrs in insect viruses has been reported. Sequences within 64% identity with an AcMNPV hr have been recognized as hr sequences with as little as 64% identity with an AcMNPV hr have been recognized as hr sequences from Bombyx mori Nuclear Polyhedrosis Virus (Majima, K., et al., J. Virol. 67, 7513-7521, 1993).

It has been reported that baculovirus hrs can act through an AAV2 cis-acting Rep-binding Element (RBE) to enhance AAV production in an insect cell line that harbors AAV. Binding Element (RBE) to enhance AAV production in an insect cell line that harbors AAV Rep and Cap genes (Aslanidi, G., et al., Proc. Nat'l. Acad. Sci. USA 106, 5059-5064, 2009). This study tested expression of AAV Rep genes in a transient transfection assay, and reported elevated transcription upon transfection with a construct harboring an RBE upstream to the Rep gene induced by trans-acting baculovirus expression vector-encoded immediate-early trans-regulator 1 (IE-1). In their model, one of the products (an AAV:rep:protein) interacts with the RBE to induce rescue/amplification and mediate more transcription.

Baculovirus hrs have also been reported to stabilize recombinant baculoviruses in continuous-cascade insect-cell bioreactors (Pijhan, G. P., et al., Biotechnol. Bioeng. 87, 743-753, 2004). However, there has been no report of an hr sequence enhancing AAV virus productivity in a baculovirus system.

Summary

The present inventor has developed compositions and methods for enhancing adenovirus (AAV) production in baculovirus-based systems in insect cells in vitro. The associated virus (AAV) production in baculovirus-based systems in insect cells in vitro. The compositions and methods employ vectors that do not have a AAV Rep-binding element (RBE) but include a baculovirus homologous region (hr). In various configurations, stability of an insect cell line harboring a baculovirus genome which includes an AAV genome can be maintained over multiple passages.

In various configurations, AAV production in insect cells comprising a vector of the present teachings can be enhanced compared to vectors that do not include an hr. The present teachings can be enhanced compared to vectors that do not include an hr.
Furthermore, insect cell lines comprising a vector of the present teachings can stably maintain high titers of AAV production even after repeated passages of vectors. In various embodiments, the present teachings include vectors. In various configurations, a vector can comprise a baculovirus or a plasmid, such as, for example but without limitation, a bacmid shuttle vector (Luckow, V., et al., J. Virol. 67, 4566-4579, 1993). In various configurations, a vector can comprise an AAV Cap expression cassette, an AAV Rep expression cassette, and a baculovirus homologous region (hr). In various configurations, the hr can be located up to about 4 kb from a start codon of an AAV expression cassette. In hr can be located up to about 4 kb from a start codon of an AAV expression cassette. In various configurations, the hr can be an insertion. In various configurations, the hr can be an insertion. In various configurations, the hr can be located up to about 0.5 kb, about 1 kb, about 1.5 kb, about 2 kb, about 2.5 kb, about 3 kb, about 3.5 kb, about 4 kb, about 4.5 kb from a start codon of an AAV expression cassette. In AAV can be produced in insect cells at using vectors that comprise a hr but do not comprise an RBE.
In various configurations, a vector of the present teachings can comprise, in 5' to 3' order, a Rep expression cassette, a Cap expression cassette, and a baculovirus homologous region.

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In various configurations, a vector of the present teachings can comprise, in 5' to 3' order, a baculovirus homologous region, a Cap expression cassette, and a Rep expression cassette.

In various configurations, the hr region can be located between the Rep expression cassette and the Cap expression cassette. In various configurations, the Rep expression cassette and the Cap expression cassette can be in a head-to-head (5' to 5') orientation, a tail-to-tail (3' to 3') orientation, or a head-to-tail (5' to 3') orientation.

In various configurations, the present teachings include insect cell lines, such as, for example and without limitation, Sf9 cells, Tni Pro cells, or E4a cells that comprise a vector described herein. In various configurations, the present teachings include insect cell lines, described herein. In various configurations, the present teachings include insect cell lines, such as and without limitation, Sf9 cells.

In some embodiments, the present teachings include methods of growing baculovirus in vitro. In various configurations, these methods include providing a culture of insect cells, infecting the insect cells with a vector comprising an AAV Rep expression cassette, an AAV Cap expression cassette, and a baculovirus homologous region (hr), and incubating the cells. In various configurations, the hr can be up to about 4 kb from an AAV expression cassette. In various configurations, the hr can be up to about 4 kb from an RepV expression cassette. In various aspects, resulting cell lines can be repeatedly passaged. In various aspects, resulting cell lines can be repeatedly passaged.
In some embodiments, the present teachings include methods of growing AAV in vitro. In some embodiments, the present teachings include methods of growing AAV in vitro. In these methods, a baculovirus comprising an AAV Rep cassette and an hr can be used to infect or transfect (or co-infect or co-transfect) an insect cell line comprising an AAV Cap cassette, an AAV Rep cassette and an hr, can be grown in vitro from a cell line comprising a vector comprising an AAV Cap expression cassette, an AAV Rep expression cassette and an hr, and a cell line comprising a vector comprising an AAV Rep expression cassette and an AAV Cap expression cassette, and an AAV Rep expression cassette but no hr after 7 passages. In various configurations, AAV production from an insect cell line comprising a vector comprising an AAV Rep expression cassette and an AAV Cap expression cassette, an AAV Rep expression cassette and an hr of the present teachings can be, e.g., after 7 passages, at least 2-fold greater; at least 3-fold greater, or at least 4-fold greater than that of an insect cell line comprising a vector comprising an AAV Rep expression cassette and an AAV Cap expression cassette, and an AAV Rep expression cassette but no hr after 8 passages. In various configurations, AAV production from an insect cell line comprising a vector comprising an AAV Rep expression cassette, an AAV Rep expression cassette and an hr of the present teachings can be, e.g., after 7 passages, at least 2-fold greater, at least 3-fold greater, or at least 4-fold greater than that of an insect cell line comprising a vector comprising an AAV Rep expression cassette and an AAV Cap expression cassette, an AAV Rep expression cassette and an hr of the present teachings can be, e.g., after 10 passages, at least 2-fold greater, at least 3-fold greater, or at least 4-fold greater than that of an insect cell line comprising a vector comprising an AAV Rep expression cassette and an AAV Cap expression cassette but no hr after 9 passages. In various configurations, AAV production from an insect cell line comprising a vector comprising an AAV Rep expression cassette and an AAV Cap expression cassette, an AAV Rep expression cassette and an hr of the present teachings can be, e.g., after 10 passages, at least 2-fold greater, at least 3-fold greater, or at least 4-fold greater than that of an insect cell line comprising a vector comprising an AAV Rep expression cassette and an AAV Cap expression cassette, and an AAV Rep expression cassette but no hr after 8 passages. In some embodiments, the present teachings include methods of growing baculovirus in vitro. In these methods, baculovirus vectors comprising AAV Cap and AAV Rep cassettes can be used to infect or transfect (or co-infect or co-transfect) an insect cell line comprising an AAV Rep expression cassette, and an AAV Cap expression cassette but no hr after 9 passages. In various configurations, AAV production from an insect cell line comprising a vector comprising an AAV Rep expression cassette and an AAV Cap expression cassette, and an AAV Rep expression cassette but no hr after 8 passages. In some embodiments, the present teachings include methods of growing AAV in vitro. In some embodiments, the present teachings include methods of growing AAV in vitro. In these methods, a baculovirus comprising an AAV Rep cassette can be used to infect or transfect (or co-infect or co-transfect) an insect cell line comprising an AAV Rep expression cassette, and an AAV Cap expression cassette but no hr after 9 passages.
Trichoplusia ni or E4a cells from Estima acrea. In various configurations, the cell can be co-
Trichoplusia ni or E4a cells from Estima acrea. In various configurations, the cell can be co-
infect ed with a transgene between inverted terminal repeats (ITRs) of AAV, such as, without limitation, ITRs of AAV5. In some configurations, a transgene can be a reporter gene, such as, for example and without limitation, a gene encoding a green fluorescent protein, or a red fluorescent protein. An infected (or transfected) cell can be grown in vitro and thereby form a cell line that produces baculovirus. Such cell lines can be repeatedly passaged. In various configurations, the titer of baculovirus comprising an AAV gene such as a Cap gene or a Rep gene can be at least 20% of the titer of total baculovirus at passage P7 when the vector includes an hr. In various configurations, the titer of baculovirus comprising an AAV gene such as a Cap gene or a Rep gene can be at least 30% of the titer of total baculovirus at passage P7 when the vector includes an hr. In various configurations, the titer of baculovirus comprising an AAV gene such as a Cap gene or a Rep gene can be at least 40% of the titer of total baculovirus at passage P7 when the vector includes an hr. In various configurations, the titer of baculovirus comprising an AAV gene such as a Cap gene or a Rep gene can be at least 50% of the titer of total baculovirus at passage P7 when the vector includes an hr. In various configurations, the titer of baculovirus comprising an AAV gene such as a Cap gene or a Rep gene can be at least 70% of the titer of total baculovirus at passage P7 when the vector includes an hr. In various configurations, the titer of baculovirus comprising an AAV gene such as a Cap gene or a Rep gene can be at least 90% of the titer of total baculovirus at passage P7 when the vector includes an hr. In various configurations, the titer of baculovirus comprising an AAV gene such as a Cap gene or a Rep gene can be 100% of the titer of total baculovirus at passage P7 when the vector includes an hr. In various configurations, the titer of baculovirus comprising an AAV gene such as a Cap gene or a Rep gene can be greater than 5% of the titer of total baculovirus at passage P10 when the vector includes an hr.
AAV at passage 7 (P7) from an insect cell line harboring a control vector without an hr can be less than 60% of AAV yield from an insect cell line harboring a vector that has an hr. In some configurations, relative yield of AAV at passage 7 (P7) from an insect cell line harboring a control vector without an hr can be less than 50% of AAV yield from an insect cell line harboring a vector that has an hr. In some configurations, relative yield of AAV at passage 7 (P7) from an insect cell line harboring a control vector without an hr can be less than 40% of AAV yield from an insect cell line harboring a vector that has an hr. In some configurations, relative yield of AAV at passage 7 (P7) from an insect cell line harboring a control vector without an hr can be less than 20% of AAV yield from an insect cell line harboring a vector that has an hr.

The present teachings include, without limitation, the following aspects:

1. A baculovirus vector comprising:
   - an AAV Cap expression cassette;
   - an AAV Rep expression cassette; and
   - a baculovirus homologous region (hr) located up to about 4 kb from a start codon of an AAV expression cassette.

2. A vector in accordance with aspect 1, comprising, in 5' to 3' order, the Cap expression cassette, the Rep expression cassette, and the baculovirus homologous region (hr).

3. A vector in accordance with aspect 1, comprising, in 5' to 3' order, the Rep expression cassette, the Cap expression cassette, and the baculovirus homologous region (hr).

4. A vector in accordance with aspect 1, comprising, in 5' to 3' order, the Cap expression cassette, the baculovirus homologous region (hr), and the Rep expression cassette.

5. A vector in accordance with aspect 1, comprising, in 5' to 3' order, the Rep expression cassette, the baculovirus homologous region (hr), the Cap expression cassette, and the baculovirus homologous region (hr).

6. A vector in accordance with aspect 1, comprising, in 5' to 3' order, the baculovirus homologous region (hr), the Cap expression cassette, and the Rep expression cassette.
8. A vector in accordance with aspect 1, wherein the hr region is between the Rep expression cassette and the Cap expression cassette, and wherein the Rep expression cassette and the Cap expression cassette are in a head-to-head (5' to 5') orientation.

9. A vector in accordance with any one of aspects 1-9, wherein the baculovirus homologous region is an hr sequence.

10. A vector in accordance with any one of aspects 1-9, wherein the vector is exclusive of a Rep binding element (RBE).

11. An insect cell line comprising cells expressing a vector in accordance with any one of aspects 1-10.

12. An insect cell line in accordance with aspect 11, wherein the cells further comprise a second vector, said second vector comprising a transgene flanked by AAV ITRs.

13. A method of producing baculovirus in vitro, comprising: providing a culture of insect cells in accordance with aspect 11 or aspect 12, and incubating the cells.

14. A method in accordance with aspect 13, wherein the incubating the cells comprises passaging the cells, and wherein AAV production yield at passage 7 is at least 2-fold greater compared to a control insect cell line comprising a baculovirus vector comprising an AAV Cap expression cassette and an AAV Rep expression cassette but not baculovirus hr.

15. A method in accordance with aspect 13, wherein the titer at passage 7 of baculovirus comprising the AAV Cap expression cassette is greater than 21.5% of total baculovirus titer (as measured by qPCR of gp64 for total BV and of Cap for AAV).

16. A method of producing AAV in vitro, comprising: providing a culture of insect cells, infecting or transfecting the insect cells with a baculovirus vector in accordance with any one of aspects 1-10, and incubating the cells.

17. A method in accordance with aspect 16, wherein the yield at P7 of AAV from the insect cells is at least 50% greater than the yield at P7 of AAV from insect cells comprising a baculovirus hr at least 50% greater than the yield at P7 of AAV from insect cells comprising a baculovirus vector without the hr.

18. A method in accordance with aspect 16, wherein the yield at P7 of AAV from insect cells comprising a baculovirus hr is at least 20% greater than the yield of AAV from cells comprising a baculovirus hr.

19. A method in accordance with aspect 16, wherein the baculovirus vector is exclusive of a Rep binding element (RBE).

20. A method of producing AAV in vitro, comprising growing an insect cell culture comprising a vector of any one of aspects 1-10, and a vector comprising a transgene flanked by AAV ITRs, a vector of any one of aspects 1-10, and a vector comprising a transgene flanked by AAV ITRs.
21. A baculovirus vector without a Rep binding element (RBE) for producing AAV in insect cells in vitro, comprising an AAV, Cap expression cassette, an AAV, Rep expression cassette, and a baculovirus homologous region (hr) located up to about 4 kb of a start codon of an AAV expression cassette.

Brief Description of the Drawings

FIG. 1 illustrates a baculovirus shuttle plasmid of the present teachings, comprising an hr2 sequence between an AAV8 capsid gene and an AAV2 rep gene.

FIG. 2 illustrates a baculovirus shuttle plasmid of the present teachings, comprising an AAV8 Cap gene and an hr2 sequence between an AAV rep gene and a gentamicin resistance (GmR) gene.

FIG. 3 illustrates a baculovirus shuttle plasmid of the present teachings, comprising an AAV9 Cap gene and an hr2 sequence between an AAV rep gene and a gentamicin resistance (GmR) gene.

FIG. 4 illustrates a baculovirus shuttle plasmid of the present teachings, comprising an AAV6 Cap gene and an hr2 sequence between an AAV rep gene and a gentamicin resistance (GmR) gene.

FIG. 5 illustrates a baculovirus shuttle plasmid of the present teachings, comprising an AAV1 Cap gene and an hr2 sequence between an AAV rep gene and a gentamicin resistance (GmR) gene.

FIG. 6 illustrates a baculovirus shuttle plasmid of the present teachings, comprising an AAV5 Cap gene and an hr2 sequence between an AAV rep gene and a gentamicin resistance (GmR) gene.

FIG. 7 illustrates the GFP expression cassette flanked by two ITRs in baculovirus shuttle plasmid V372-pFB-CMV-GFP-SV40pA-full ITR.

FIG. 8 illustrates comparative AAV production yields by recombinant baculoviruses (rBVs) with or without the hr2 sequence.

FIG. 9A-H illustrates comparative recombinant baculovirus (rBV) titers with or without the hr2 sequence from passage 3 to passage 10.
FIG. 10A-E illustrates comparative AAV production yields between recombinant baculoviruses (rBVs) with or without the hr2 sequence from passage 3 to passage 10 or at passage 10 between AAV strains.

FIG. 11A-B illustrates Western blot expression of AAV capsid proteins in cells infected with recombinant baculoviruses with and without hr2 sequences.

FIG. 12 A-B illustrates Western blot expression of AAV rep proteins in cells infected with recombinant baculoviruses with and without hr2 sequences.

Detailed Description


The following materials and methods are also used in various aspects of the present teachings.

Insect cell culture

Spodoptera frugiperda Sf9 cells, Trichoplusia ni cells, and Estima acrea cells were cultured in corn storage bottles at 28°C in ESF921 medium (Expression Systems) supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin (Mediatech). The cells were split 1:4 once the cell density reaches 8 x 10^6 cells/ml for maintenance.

Plasmid construction and recombinant baculovirus generation

The hr2 sequence was PCR amplified from the baculovirus genome and cloned into plasmid V053-pFBD-inRepOpt-inCap8 using T059-pFBD-inRepOpt-hr2-inCap8. In order to include the kozak sequence, the BstZ17I-AgeI fragment was replaced with the BstZ17I-AgeI fragment with kozak sequence.
upstream of VP1 start codon from V150-pFB-inCap8-inRep-kozak to create V277-pFB-n inCap8-hr2-inRep-kozak where the hr2 is located between the Rep and the Cap expression cassettes. To insert the hr2 sequence into another location after the polyA sequence of the Rep expression cassette, the hr2 sequence was PCR-amplified with primers 3205F (5'-GCTTTACGATAGAATTCTACGTTAGT-3'), SEQ ID NO: 7) and 3206R (5'-GGCTACTAGTGGTATACGTTAATTGACACTCGT-3'), SEQ ID NO: 8) from V277.

The pc promoter sequence was amplified with primers 3065F (5'-ATTTCACTTTATGACAGGGCCGGT-3') and 3204R (5'-GAATTTCTACGTTGAAGCCAGGAGATACGTTACGCTACTCGT-3'), SEQ ID NO: 9) and 3204R (5'-GAATTTCTACGTTGAAGCCAGGAGATACGTTACGCTACTCGT-3'), SEQ ID NO: 10) from V150. These hr2 and pc promoter PCR fragments were joined together through a second PCR reaction with primers 3065F and 3206R. The joined PCR fragment was digested with BsrGI and SnaBI and ligated into the BsrGI and SnaBI sites of V150, V277, V289, and V146 to create V288-pFB-inCap8-inRep-hr2, V289-pFB-inCap9-inRep-hr2, V290-pFB-inCap6-inRep-hr2, V291-pFB-inCap1-inRep-hr2, and V295-pFB-inCap5-inRep-hr2 respectively. Examples of plasmids with an hr sequence insertion are illustrated in FIGS. 11–66.

Plasmid pFB-CMV-GFP was constructed by PCR amplifying the GFP fragment which was then cloned into the multiple cloning sites of V032-pFB-CMV-SV40pA (FIG. 77) according to manufacturer's protocol (Invitrogen). Briefly, plasmids were diluted to a concentration of 2 ng/ul and 2 ul of plasmid DNA was used to transform DHIOBac competent cells. After 2 days of incubation, white colonies were picked and miniprep bacmid DNAs were prepared. The miniprep bacmid DNAs were used to transfect Sf9 cells to generate recombinant baculoviruses.

Plaque purification and passaging of recombinant baculovirus

The generated recombinant baculoviruses were plaque purified in order to get homogenous clones. Briefly, Sf9 cells were plated on 6-well plates with cell density of 1x10^6 cells/well in 2 ml ESF921 media and incubated at 28°C for 30 min. The baculoviruses were each diluted to 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 in 1 ml volume. At the end of incubation, media were removed from the wells and 250 µl of each dilution was added to infect the Sf9 cells at 28°C for 1 hr. At the end of the incubation, 1 ml of 1% agarose overlay (cooled down to 4°C) was added to the wells. When the agarose had solidified, 2 ml of ESF921 media was added to each well and the plates were incubated at 28°C for 7 days. Well-formed plaques were picked and used to infect insect cells for passaging, to 7 days. Well-formed plaques were picked and used to infect insect cells for passaging.
To passage the plaque purified recombinant baculoviruses, insect cells were infected with 5 moil of the viruses for 3 days at 28°C and, supernatant was harvested. The harvested viruses were used to infect fresh insect cells again and so on until passage 10.

Real-time quantitative PCR (qPCR) quantification of recombinant baculoviruses and AAV

Real-time quantitative PCR (qPCR) quantification of recombinant baculoviruses and AAV vectors

To determine the titers of recombinant baculoviruses and AAV, a qPCR method was employed. It was empirically determined that one plaque-forming unit (pfu) comprises about 20 genome copies of the virus. Briefly, harvested viruses were diluted in qPCR dilution buffer and heated to 95°C for 30 min to break the virus particles. The treated virus particles were then assayed in the CHROMO™ system (Bio-Rad Laboratories, Inc., Hercules, CA) together with a known standard. The Ct values were converted to pfu and used to guide the baculovirus passage and AAV production.

AAV vector production and quantification

Recombinant baculoviruses were used to infect insect cells to produce AAV vectors. Briefly, 5 moil of recombinant baculovirus containing the Rep and Cap genes with or without the hr2 sequence were co-infected with 5 moil of recombinant baculovirus containing the GFP marker gene flanked by AAV ITRs, for 3 days at 28°C. Cell pellets were collected by centrifugation at 3000 rpm for 10 min. The cell pellets were lysed in SF9 lysis buffer (50 mM Tris-HCl, pH 7.8, 50 mM NaCl, 2 mM MgCl₂, 1% Sarkosyl, 1% Triton X-100, and 140 units/ml Benzonase) by sonication. Cell debris was removed by centrifugation at 8000 rpm for 20 min. The cleared lysates were used for quantification of AAV productivity as follows: the 20 min cleared lysates were used for quantification of AAV productivity as follows: the lysates were diluted with qPCR dilution buffer and contaminating DNA was destroyed by incubating with DNase I enzyme at 37°C for 1 h. The DNase I enzyme was inactivated by heating at 95°C for 30 min. in the presence of 100 mM EDTA. The treated AAV samples were further diluted and assayed in the Chromo™ qPCR machine. The Ct values were converted to AAV vector genome copies.

Western blot analysis

Recombinant baculoviruses containing the rep and cap genes were used to infect Sf9 cells for 3 days and cell pellets were harvested by centrifugation at 30,000 rpm for 30 min. The cell pellets (with about 2 × 10⁶ cells) were first resuspended in 100 ul of PBS buffer and then vortexed with 100 ul of 4xLDS sample buffer to lyse the cells. The lysates were heated at 95°C for 30 min.

AAV vector genome copies.

Western blot analysis

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95°C for 5 min and then sonicated for 20 seconds to shear the genomic DNA. After brief centrifugation, the lysates were then loaded onto a 10% SDS-gel to separate the proteins. The proteins were then transferred from the gel to nitrocellulose membranes. After blocking with 5% skim milk, the membranes were probed with specific antibodies against the rep or cap proteins. A second antibody coupled with horseradish peroxidase (HRP) against the first antibody was used to detect the rep or cap proteins through color matrix reaction.

Examples

The present teachings including descriptions provided in the Examples that are not intended to limit the scope of any claim or aspect. Unless specifically presented in the past tense, an example can be a prophetic or an actual example. The following non-limiting examples are provided to further illustrate the present teachings. Those of skill in the art, in light of the present disclosure, will appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the present teachings.

Example 1

This example illustrates a shuttle vector of the present teachings.

In this plasmid (FIG. 1), an hr2 sequence is located between an AAV8 capsid gene cassette and an AAV2 rep gene cassette. The vector includes the Cap and Rep genes in head-to-head orientation. This shuttle plasmid was used to generate recombinant baculovirus (rBV), which in turn was used to produce AAV8 in insect cells.

Example 2

This example illustrates a shuttle vector of the present teachings.

In this plasmid (FIG. 2), an hr2 sequence is located between an AAV2 rep gene cassette and a gentamicin resistance (GmR). The vector also includes an AAV8 capsid gene cassette and a gentamicin resistance (GmR). The vector also includes an AAV8 capsid gene cassette. The Cap and Rep genes are in a head-to-tail orientation. This plasmid was used to generate recombinant baculovirus (rBV), which in turn was used to produce AAV8 in insect cells.

Example 3

This example illustrates a shuttle vector of the present teachings.

This example illustrates a shuttle vector of the present teachings.
In this plasmid (FIG. 3), an hr2 sequence is located between an AAV2 rep gene cassette and a gentamicin resistance (GmR). The vector also includes an AAV9 capsid gene cassette. The Cap and Rep genes are in a head-to-tail orientation. This plasmid was used to generate recombinant baculovirus (rBV), which in turn was used to produce AAV9 in insect cells.

Example 4

This example illustrates a shuttle vector of the present teachings.

In this plasmid (FIG. 4), an hr2 sequence is located between an AAV2 rep gene cassette and a gentamicin resistance (GmR). The vector also includes an AAV6 capsid gene cassette and a gentamicin resistance (GmK). The vector also includes an AAV6 capsid gene cassette. The Cap and Rep genes are in a head-to-tail orientation. This plasmid was used to generate recombinant baculovirus (rBV), which in turn was used to produce AAV6 in insect cells.

Example 5

This example illustrates a shuttle vector of the present teachings.

In this plasmid (FIG. 5), an hr2 sequence is located between an AAV2 rep gene cassette and a gentamicin resistance (GmR). The vector also includes an AAV1 capsid gene cassette. The Cap and Rep genes are in a head-to-tail orientation. This plasmid was used to generate recombinant baculovirus (rBV), which in turn was used to produce AAV1 in insect cells.

Example 6

This example illustrates a shuttle vector of the present teachings.

In this plasmid (FIG. 6), an hr2 sequence is located between an AAV2 rep gene cassette and a gentamicin resistance (GmR). The vector also includes an AAV5 capsid gene cassette and a gentamicin resistance (GmK). The vector also includes an AAV5 capsid gene cassette. The Cap and Rep genes are in a head-to-tail orientation. This plasmid was used to generate recombinant baculovirus (rBV), which in turn was used to produce AAV5 in insect cells.

Example 7

This example illustrates that an hr sequence enhances yields of AAV production.

In these experiments, the hr2 sequence was cloned either between the Rep and the Cap expression cassettes in plasmid V277 (FIG. 1) or after the poly A sequence of the Rep Cap expression cassettes in plasmid V277 (FIG. 1) or after the poly A sequence of the Rep
expression cassette in plasmids V289 (FIG. 3) and V295 (FIG. 6) and recombinant baculoviruses were prepared. These recombinant baculoviruses were used to coinfect Sf9 cells with recombinant baculovirus carrying GFP gene for AAV production. The results are shown in FIG. 8. The data indicate that hr2 sequence enhances the AAV productivity. The increase of AAV productivity ranged from 2- to 4-fold compared to controls lacking an hr.

Example 8

This example illustrates that an hr sequence enhances stability of recombinant baculoviruses containing the AAV rep and cap genes. In these experiments, to further analyze the stability of recombinant baculoviruses with or without the hr2 sequence, plaque purification and passaging of the recombinant baculoviruses multiple times were employed. A pair of qPCR primers—gp64F (5’-CCCTCTGTGATCTTGCTCT AACG-3’ SEQ ID NO: 11) and gp64R (5’-CGGTGAAACGCXAAGTCGAGC ACCG-3’ SEQ ID NO: 12)—corresponding to the gp64 gene (present in all recombinant baculoviruses of the present teachings) was used to determine total baculovirus titer. For baculoviruses comprising the Rep and Cap expression cassettes, a pair of qPCR primers—Rep2F (5’-ATTCCATGCTCCACCTCA ACC-3’ SEQ ID NO: 13) and Rep2R (5’-GCCGTCTGGATCATG ACTTT-3’ SEQ ID NO: 14)—corresponding to the Rep sequence, was used to determine titer of these baculoviruses.

Recombinant baculoviruses carrying Cap1-Rep (FIG. 5), Cap6-Rep (FIG. 4), Cap9-Rep (FIG. 3), and Cap8-Rep (FIG. 2) were selected for these experiments. For each passage, the total baculovirus pfu was determined with the gp64 primers, and set to 100%. The specific total baculovirus pfu was determined with the gp64 primers, and set to 100%. The specific total baculovirus pfu for each passage was determined using the Rep primers set as percentage of total baculovirus. The results are shown in FIG. 9A-H for recombinant baculovirus (rBV) titers baculovirus. The results are shown in FIG. 9A-H for recombinant baculovirus (rBV) titers with or without the hr2 sequence. FIG. 9A illustrates rBV titers determined with gp64 and with or without the hr2 sequence. FIG. 9B illustrates rBV titers determined with gp64 and rep2 qPCR primers in cells harboring rBV in Cap1-inRep (CapiV188) expressing AAV1 capsid and AAV2 rep genes without the hr2 sequence. FIG. 9C illustrates rBV titers determined with gp64 and rep2 qPCR primers in cells harboring rBV in Cap1-inRep-hr2 (CapiV291 expressing AAV1 capsid and AAV2 rep genes with the hr2 sequence). FIG. 9D illustrates rBV titers determined with gp64 and rep2 qPCR primers in cells harboring rBV in Cap6-inRep (Cap6 V195) expressing AAV6 capsid and AAV2 rep genes with the hr2 sequence.
FIG. 9E illustrates rBV titers determined with gp64 and rep2 qPCR primers in cells harboring rBV-inCap9-inRep (Cap9,V212), expressing AAV9 capsid and AAV2 rep genes, without hr2 sequence. FIG. 9F illustrates rBV titers determined with gp64 and rep2 qPCR primers in cells harboring rBV-inCap9-inRep-hr2 (Cap9,V289), expressing AAV9 capsid and AAV2 rep genes with hr2 sequence. FIG. 9G illustrates rBV titers determined with gp64 and rep2 qPCR primers in cells harboring rBV-Cap8-inRep (Cap8,V150) expressing AAV8 capsid and AAV2 rep genes without hr2 sequence. FIG. 9H illustrates rBV titers determined with gp64 and rep2 qPCR primers in cells harboring rBV-Cap8-inRep-hr2 (Cap8,V288) expressing AAV8 capsid and AAV2 rep genes with hr2 sequence. rBVs were produced and passaged in AAV8 capsid and AAV2 rep genes with hr2 sequence. rBVs were produced and passaged in Sf9 cells (FIG. 9A-D), Tni pro cells (FIG. 9E-F), and E4a cells (FIG. 9G-H). The data indicate that specific baculovirus titer decreased with increasing number of passages when the baculovirus contained the hr2 sequence (FIG. 9A, FIG. 9E for Sf9 cells, FIG. 9D for Tni pro cells, FIG. 9F for E4a cells), whereas the baculovirus did not contain all hr2 sequence (FIG. 9A, FIG. 9C for AAV1 or AAV6, FIG. 9E for AAV2, and FIG. 9G for AAV8), regardless of whether Sf9 (FIG. 9A-D), Tni Pro (FIG. 9E-F), or E4a cells (FIG. 9G-H) were the host cells. By passage P10, baculovirus with AAV Capl or AAV/Cap6 were below 10% of total rBV titer when the vectors contained all hr2 (FIG. 9A, FIG. 9C, FIG. 9E, and FIG. 9G), but baculovirus with AAV/Cap1 or AAV/Cap6 were about 20% of total rBV titer when the vectors contained hr2 (FIG. 9 B, FIG. 9 D, FIG. 9 F, FIG. 9 H).

Example 9

This example illustrates that an hr sequence enhances AAV productivity of recombinant baculoviruses (rBV) containing the AAV rep and cap genes. In these experiments, the rBVs with or without the hr sequence from passage 3 to passage 10 were used to co-infect Sf9, Tni Pro, and E4a cell lines with the rBV containing passage 10 were used to co-infect Sf9, Tni Pro, and E4a cell lines with the rBV containing AAV1 in Sf9 cells (FIG. 10A), AAV6 in Sf9 cells (FIG. 10B), AAV9 in Tni pro cells (FIG. 10C) and AAV8 in E4a cells (FIG. 10D) was maintained over passages 3-10 when the baculovirus vector included an hr2 sequence. In contrast, production yield of AAV1, AAV6, AAV9, and AAV8 declined dramatically over passages 3-10 in the absence of hr2 in the rBV. To further confirm this observation, Sf9 cells were co-infected with passage 10 rBVs with rBV hr2 (V290, V288, and V289) or without hr2 (V195, V150, and V212) sequence to produce hr2 (V290, V288, and V289) or without hr2 (V195, V150, and V212) sequence to produce.
AAV6 (V195 and V290), AAV8 (V150 and V288), and AAV9 (V212 and V289) vectors. The results show substantially higher AAV production yields from SF9 cells infected with rBV containing hr2 than those infected with rBV without hr2 sequence (FIG. 10E) (compare hr+ rBVs V290, V288 and V289, to hr rBVs V195, V150 and V212).

Example 10

This example illustrates that AAV rep and cap expression directly correlates with rBV stability through multiple passages.

In these experiments, Western blots were performed to determine the expression level of rep and cap proteins. FIG. 11A-B illustrate the expression of AAV6 capsid proteins VP1, VP2, and VP3 after infection of SF9 cells with recombinant baculoviruses rBV-inCap6-inRep (V195) without hr2 sequence (FIG. 11A) and rBV-inCap6-inRep-hr2 (V290) with hr2 sequence (FIG. 11B) from passage 3 to passage 10 respectively. M, protein size markers; lanes 1-8, cell lysates prepared from SF9 cells infected with rBVs from passages 3 to 10.

FIG.12A-B illustrate the expression of AAV2 rep proteins REP78 and REP52 after infection of SF9 cells with recombinant baculoviruses rBV-inCap8-inRep (V150) without hr2 sequence (FIG. 12A) and rBV-inCap8-inRep-hr2 (V288) with hr2 sequence (FIG. 12B). M, protein size markers; lanes 1-5, cell lysates prepared from SF9 cells infected with rBVs from passages 6 to 10. The results in FIG. 11 and FIG. 12 indicate that rBVs with an hr sequence express higher levels of rep and cap proteins throughout multiple passages, including later passages, compared to rBVs lacking an hr sequence.

All cited references are incorporated by reference, each in its entirety. Applicant reserves the right to challenge any conclusions presented by the authors of any reference.
What is claimed is:

1. A baculovirus vector comprising:
   an AAV Cap expression cassette;
   an AAV Rep expression cassette; and
   a baculovirus homologous region (hr) located up to about 4 kb from a start codon of
   an AAV expression cassette.

2. A vector in accordance with claim 1, comprising, in 5' to 3' order, the Cap expression
   cassette, the Rep expression cassette, and the baculovirus homologous region (hr).

3. A vector in accordance with claim 1, comprising, in 5' to 3' order, the Rep expression
   cassette, the Cap expression cassette, and the baculovirus homologous region (hr).

4. A vector in accordance with claim 1, comprising, in 5' to 3' order, the Cap expression
   cassette, the baculovirus homologous region (hr), and the Rep expression cassette.

5. A vector in accordance with claim 1, comprising, in 5' to 3' order, the Rep expression
   cassette, the baculovirus homologous region (hr), and the Cap expression cassette.

6. A vector in accordance with claim 1, comprising, in 5' to 3' order, the baculovirus
   homologous region (hr), the Cap expression cassette, and the Rep expression cassette.

7. A vector in accordance with claim 1, comprising, in 5' to 3' order, the baculovirus
   homologous region (hr), the Rep expression cassette, and the Cap expression cassette.

8. A vector in accordance with claim 1, wherein the hr region is between the Rep expression
   cassette and the Cap expression cassette, and wherein the Rep expression cassette and the
   Cap expression cassette are in a head to head (5' to 5') orientation.

9. A vector in accordance with any one of claims 1-9, wherein the baculovirus homologous
    region is an hr2 sequence.

10. A vector in accordance with any one of claims 1-9, wherein the vector is exclusive of a
    Rep binding element (RBE).

11. An insect cell line comprising cells comprising a vector in accordance with any one of
    claims 1-10, comprising cells comprising a vector in accordance with any one of
    claims 1-10.
12. An insect cell line in accordance with claim 11, wherein the cells further comprise a second vector, said second vector comprising a transgene flanked by AAV ITRs.

13. A method of growing baculovirus in vitro, comprising:
   providing a culture of insect cells in accordance with claim 11 or claim 12; and
   incubating the cells.

14. A method in accordance with claim 13, wherein the incubating the cells comprises
   passaging the cells, and wherein AAV production yield at passage 7 is at least 2-fold greater
   compared to a control insect cell line comprising a baculovirus vector comprising an AAV
   Cap expression cassette and an AAV Rep expression cassette but no baculovirus hr.

15. A method in accordance with claim 13, wherein the titer at passage 7 of baculovirus
   comprising the AAV Cap expression cassette is greater than 21.5% of total baculovirus titer.

16. A method of growing AAV in vitro, comprising:
   providing a culture of insect cells;
   infecting or transfecting the insect cells with a baculovirus vector in accordance with
   anyone of claims 1-10; and
   incubating the cells.

17. A method in accordance with claim 16, wherein the yield at P7 of AAV from insect
   cells is at least 20% greater than the yield of AAV from cells comprising a baculovirus vector without the hr.

18. A method in accordance with claim 16, wherein the yield at P7 of AAV from cells
   comprising the baculovirus hr is at least 20% greater than the yield of AAV from cells
   comprising a baculovirus vector without the hr.

19. A method in accordance with claim 16, wherein the baculovirus vector is exclusive of a Rep binding element (RBE).

20. A method of producing AAV in vitro, comprising growing an insect cell culture
   comprising a vector of any one of claims 1-10, and a vector comprising a transgene flanked
   by AAV ITRs, a vector of any one of claims 1-10, and a vector comprising a transgene flanked
   by AAV ITRs.

21. A baculovirus vector without a Rep binding element (RBE) for producing AAV in insect
   cells in vitro, comprising:
   a baculovirus vector without a Rep binding element (RBE) for producing AAV in insect
cells comprising:
   an AAV Cap expression cassette;
an AAV Rep expression cassette; and
an AAV Rep expression cassette; and located up to about 4 kb from a start codon of
an AAV baculovirus homologous region (hr) located up to about 4 kb from a start codon of
an AAV expression cassette.
FIG. 9A

Relative Read Itter

Cap1 V188 gp64 (-) hr 100% 100% 100% 100% 100% 100% 100% 100% 100% 100%
Cap1 V188 rep (-) hr 70.10% 19.70% 16.70% 27.10% 91.30% 91.30% 106% 106% 106% 106%
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC (2017.01) A61K 35/76, C12N 15/09, C12N 15/86, C12N 5/10, C12N 15/866, C12N 15/800

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)
IPC (2017.01) C12N, A61K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Databases consulted: NCBI, Sequence Manipulation Suite, BLAST, PATENTSCOPE, Eponline, Esp@cenet, Google Patents, CAPLUS, BIOSIS, EMBASE, MEDLINE, REGISTRY, PubMed, Google Scholar, PatBase, Derwent Innovation
Search terms used: Baculovinis, baculoviridea, Parvo, vector, AAV, Cap, Rep, expression cassette, Homologous region, start codon, insect cell.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category* | Citation of document, with indication, where appropriate, of the relevant passages |
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Relevant to claim No. 1-13,16, 19-21

Further documents are listed in the continuation of Box C.

Dale of the actual completion of the International search 06 Jul 2017
Date of mailing of the international search report 20 Jul 2017

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