Efficient production of an avian adeno-associated virus vector using insect cell/baculovirus expression system

Anping Wang, Yongjuan Wang, Shuang Wu, Weiyong Zuo, Changming Guo, Weiming Hong, Shanyuan Zhu^{*}

Jiangsu Agri-animal Husbandry Vocational College, Jiangsu Key Laboratory for High-Tech Research and Development of Veterinary Biopharmaceuticals, Taizhou, 225300, China

ABSTRACT

Recombinant avian adeno-associated virus (rAAAV) is a promising gene transfer vector for avian cells. Although rAAAV can be produced by co-transfection of HEK293 cells with three plasmids, both scalability and productivity of the transient transfection method can not meet the demand for large-scale in vivo experiments. In this study, a scalable rAAAV production method was established by using insect cell/baculovirus expression system. Three recombinant baculoviruses, namely BacARep, BacAVP and BacAGFP, were generated by transfection of Sf9 cells with the three plasmids expressing AAV Rep genes, modified VP gene or the inverted terminal repeats-flanked green fluorescent protein (GFP) gene. After demonstration of the correct expression of AAV genes, rAAAV-GFP was produced by triple infection of insect cells or triple transfection of HEK293 cells for comparison purpose. Electron microscopy revealed the formation of typical AAV particles in the insect cells. Western blotting showed the correct assembly of rAAAV particles with a VP protein ratio similar to that of AAV. Quantitative PCR showed that the insect cell-produced rAAAV yield was almost 25-fold higher than that produced by HEK293 cells. Fluorescent microscopy showed that the insect cell-produced rAAAV could transfer GFP reporter gene into two avian cell types with similar transfer efficiency to

23 that of HEK293 cell-produced rAAV. These data suggest that insect cell/baculovirus expression
24 system could be used for scalable production of rAAV, and the viral vector produced could be used as
25 the gene transfer vehicle for avian cells.

26 *Key words:* Insect cell/baculovirus expression system; Recombinant avian adeno-associated virus

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1. Introduction

Adeno-associated virus (AAV) vectors are popular gene transfer vehicles for gene therapy and vaccine development due to their quasi-absence of pathogenicity, excellent safety and long-term gene expression in a wide range of tissues (Warrington and Herzog, 2006). The traditional methods for producing recombinant AAV (rAAV) employ co-transfection of human embryonic kidney (HEK) 293 cells with the rAAV vector and helper vector, followed by infection with the helper adenovirus to induce the replication of rAAV (Samulski et al., 1987). The main drawback of this approach is the co-production of rAAV and helper virus. This problem can be overcome by replacing the helper virus with the plasmid containing necessary functions for the replication of rAAV (Matsushita et al., 1998; Salvetti et al., 1998; Xiao et al., 1998). However, the transient transfection method has limited scalability and relatively high incidence of replication competent AAV production (Galibert and Merten, 2011).

Three scalable rAAV production systems have been developed, including the use of AAV packaging and/or producer cell lines (Farson et al. 2004), use of recombinant herpes simplex virus vectors (Booth et al. 2004), and use of insect cell/baculovirus expression system (Galibert and Merten, 2011). The third system employs triple infection of insect cells with Rep-baculovirus, VP-baculovirus and AAV ITR vector genome baculovirus (Urabe et al., 2002). This system has the volumetric production yield in the range of 1.1×10^{14} vg/L, and thus is gaining in popularity in the production of viral vectors or virus-like particles (Aucoin et al., 2006; Galibert and Merten, 2011).

Avian adeno-associated virus (AAAV) was first isolated as a contamination virus from the Olson strain of quail bronchitis virus, an avian adenovirus (Yates et al., 1973). Sequence analysis shows that

AAAV shares the similar genome structure with AAV (Bossis et al., 2003; Estevez et al., 2004; Wang et al., 2011), indicating its potential use as the gene transfer vector for avian cells. Like other AAVs, recombinant AAAV (rAAAV) can be produced by co-transfection of HEK 293 cells with the AAAV vector containing gene of interest, helper vector containing *rep* and *cap* genes, and helper plasmid containing necessary functions for the replication of rAAAV (Bossis et al., 2003; Estevez et al., 2004; Wang et al., 2007). However, both scalability and productivity of the transient transfection method are limited, which restrict the in vivo use of rAAAV vectors. To overcome these limitations, in this study an efficient platform for rAAAV production was generated by using insect cell/baculovirus expression system.

2. Materials and methods

2.1. Cells and plasmid vectors

Spodoptera frugiperda Sf9 cells (Invitrogen, Germany) were grown at 27°C in Sf-900 II serum-free medium (Gibco, USA). Human embryonic kidney (HEK) 293 cells (ATCC CRL-1573, USA), chicken embryonic fibroblast DF1 cells (ATCC CRL-12203) and chicken embryonic liver (CEL) cells (ATCC CRL-2117) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal calf serum (FCS). Plasmid vector pCR-AAAV containing the complete genome of AAAV was kindly provided by Dr. J. Y. Wang at the College of Veterinary Medicine, Yangzhou University. Green fluorescent protein (GFP) reporter gene-containing AAAV transfer vector pAITR-GFP, AAAV packaging vector pcDNA-ARC and adenovirus helper vector pHelper were provided by Dr. H. Sun at the College of Veterinary Medicine, Yangzhou University.

69 2.2. Vector construction

70 AAV VP (AVP) gene was amplified by polymerase chain reaction (PCR) from pCR-AAAV
71 vector (Wang et al., 2011) using primers
72 5'-ACGGCTCTTATTTCTGACGCGATACCCGATTGGTTG-3' and
73 5'-TTACAGCGGTTTGGTGAGGTAACGGGTACCG-3'. The mutated bases relative to the wild-type
74 sequence are italicized. To express the three VP polypeptides from a single transcript in a correct ratio
75 (1:1:10) without RNA splicing, the ATG initiation codon of VP1 was mutated at position 2 to ACG to
76 reduce its translation efficiency. The downstream out-of frame ATG codon with diminished translation
77 of VP2 and VP3 was modified at position 18 to create an ACG triplet. The splice acceptor site
78 downstream of the VP1 initiation codon was mutated at position 24 to prevent the pre-mRNA splicing
79 (Urabe et al., 2002). The PCR product was subcloned into baculovirus vector pFastBac1 (Invitrogen) as
80 a *NotI/HindIII* segment, and the recombinant vector was called pFBVP. AAV Rep78 (ARep78) gene
81 was amplified using primers 5'-ATGAGGTCGTACTACGAGGTC-3' and
82 5'-TCAGCCGCAGCGTTGACTCCC-3'. The PCR product was subcloned into pFastBacDual vector
83 (Invitrogen) as a *NheI* segment, and the recombinant vector was called pFBARep78. AAV Rep52
84 (ARep52) gene was amplified using primers 5'-ATGGAGCTCGTGGATTGGCTC-3' and
85 5'-TCAGCCGCAGCGTTGACTCCC-3'. The PCR product was cloned into pFBARep78 vector as a
86 *SalI/NotI* segment, and the new recombinant vector was called pFBARep. The GFP expression cassette
87 was amplified from pEGFP-N1 vector (Clontech, USA) using primers
88 5'-TAGTTATTAATAGTAATC-3' and 5'-CTTAAGATACATTGATG-3'. The PCR product was
89 cloned into pCR-AAAV vector after removing the sequences in between the two ITRs by *PmlI/BsmBI*
90 digestion. The ITR-flanked GFP expression cassette was subcloned into pFastBacDual vector as an

EcoRI segment, and the resultant rAAAV vector was called pFBAGFP. The recombinant vectors pFBABVP, pFBABRep and pFBABGFP were used to produce recombinant baculoviruses, namely BacABVP, BacABRep and BacABGFP, by following the manufacturer's instruction of Bac-to-Bac Baculovirus Expression System (Invitrogen).

2.3. Detection of AAV Rep and VP expression in insect cells by immunofluorescence

To confirm the correct expression of AAV *rep* and *cap* genes in insect cells, Sf9 cells were seeded on 24-well plates (6×10^5 /well), incubated for 1 h, and infected with BacABRep or BacABVP (MOI 1). On day 3 post-infection, the cells were fixed for 20 min at room temperature with 4% paraformaldehyde and blocked for 60 min with 5% BSA in PBS. The standard indirect immunofluorescence was performed using mouse anti-ABRep (1:100) or anti-ABVP (1:200) serum, which was prepared by immunizing mice with *E. coli*-expressed AAV Rep or Cap protein as described previously (Li et al., 2013). After incubation with FITC-labeled goat anti-mouse IgG (1:500, Jackson ImmunoResearch, USA), the cell cultures were observed under microscope for fluorescently positive cells.

2.4. Detection of AAV Rep and VP expression by Western blotting

Sf9 cells were infected with BacABRep or BacABVP as described. The cell extracts were run on 12% SDS-PAGE and the separated proteins were transferred onto PVDF membranes (Millipore, USA) using Semi-Dry Transfer Unit (TE70X, Hoefer, USA). The membranes were blocked overnight at 4°C with 10% skimmed milk in PBST, and incubated for 2 h at room temperature with mouse anti-ABRep (1:100) or anti-ABVP (1:200). After three time washing in PBST, the membranes were incubated for 1 h with HRP-labeled goat anti-mouse IgG (1:2000, Jackson Immuno Research), and the signals were

developed with 3, 3'-diaminobenzidine substrate.

2.5. AAV vector production and purification

To produce a rAAV vector using the insect cell/baculovirus expression system, Sf9 cells were grown to 2×10^6 /ml in suspension, and infected with BacAGFP, BacAVP and BacARep (MOI 5 each). For the comparison purpose, HEK 293 cells were grown to the same density (2×10^6 /ml) and transfected with pAITR-GFP, pcDNA-ARC and pHelper as described previously (Wang et al., 2007). Three days after infection or transfection, the Sf9 cells were collected by centrifugation, and HEK 293 cells were submitted to 3 cycles of freeze-thawing before centrifugation. The resultant rAAV vector, namely rAAV-GFP, was purified by PEG precipitation method as described previously (Matsushita et al., 1998). The purified virus was titrated in triplicates by real-time PCR using *GFP*-specific primers 5'-GCTGCTGCCCCGACAACCACTACCTGAGCAC-3' and 5'-CGGCGGTACGAACTCCAGCAGGACCATGT-3' by following the instruction for iCycler Thermal Cycler (Bio-Rad, CA). Linearized pFBAGFP vector was employed as the copy standard. In addition, the rAAV-GFP was also titrated on DF1 cells as transduction units (TU)/ml as described previously (Wang et al., 2007).

2.6. Identification of rAAV vector

To identify rAAV-GFP morphologically, Sf9 cells were infected with BacAGFP, BacAVP and BacARep as described, and the cells were harvested by centrifugation for the ultrathin section preparation and rAAV purification on day 3 post-infection. The intracellular rAAV-GFP particles were observed under transmission electron microscope (UC6, Leica, Germany). The purified rAAV-GFP was strained negatively for 1 min with 2% phosphotungstic acid (pH7.4) and observed

under transmission electron microscope (Tecnai 12, FEI, USA). To identify rAAAV-GFP immunologically, the purified viral particles were analyzed by Western blotting using mouse anti-AVP serum as described.

2.7. rAAAV-mediated GFP expression in avian cells

To test the gene transfer efficiency of rAAAV-GFP produced in insect cells, DF1 cells and CEL cells were seeded on 24-well plates (1×10^5 /well) and infected with serial dilutions of rAAAV-GFP after incubation for 24 h. The cell cultures were observed directly under microscope for fluorescent-positive cells 24 h post-infection.

3. Results

3.1. Three rBacs for producing rAAAV were generated

The PCR products of ARep78 and ARep52 genes were subcloned into baculovirus expression vector pFastBacDual, in which the expression of two *rep* genes were under the control of p10 and ph promoters, respectively (Fig. 1). The recombinant vector pFBARep was transfected into Sf9 cells, and the resultant BacARep was used to infect Sf9 cells. By using anti-ARep serum as the first antibody, immunofluorescence showed that the two *Rep* genes were expressed efficiently in BacARep-infected insect cells (Fig. 2A). Furthermore, Western blotting detected the expected 80- and 55-kDa protein bands in BacARep-infected cells, but not in mock-infected cells (Fig. 2B).

To express the three AVP in insect cells and in a correct ratio, the strategy similar to BacVP vector construction (Urabe et al., 2002) was used to design BacAVP vector, including mutation of the ATG codon of VP1 into ACG to reduce its translation efficiency, mutation of the ATG codon with diminished translation of VP2 and VP3 to create an ACG triplet, and destroy of the splice acceptor site

downstream of VP1 initiation codon to prevent the pre-mRNA splicing. The PCR product of *AVP* gene was cloned into baculovirus expression vector, and the resultant pFB*AVP* vector was transfected into Sf9 cells to produce Bac*AVP*. By using anti-*AVP* serum as the first antibody, immunofluorescence showed that the *AVP* gene was expressed correctly in Bac*AVP*-infected insect cells (Fig. 2A). Moreover, Western blotting analysis showed that the three VP proteins were expressed in the expected ratio of about 1:1:10 with the expected molecular weights of 83, 66 and 60 kDa, respectively (Fig. 2C).

Both Rep and Cap genes of AAV were removed from pCR-AAV vector by restriction digestion, and the resultant ITR-containing vector was ligated to the GFP expression cassette amplified from pEGFP-N1 vector. The recombinant vector pFBAGFP was transfected into Sf9 cells and the resultant BacAGFP was harvested for infection of Sf9 cells. Fluorescent microscopy showed efficient expression of GFP in the rBac-infected cells (data not shown).

3.2. Correct rAAV is produced by triple infection of insect cells

To identify rAAV-GFP produced in insect cells, Sf9 cells were infected with BacAGFP, Bac*AVP* and BacARep, and the infected cells were collected for ultrathin section preparation and rAAV-GFP purification on day 3 post-infection. Transmission electron microscopy revealed AAV-like viral particles clustered in the nucleus of Sf9 cells (Fig. 3A). Under electron microscope, the purified viral particles had an icosahedral structure with a diameter of about 22 nm, which was morphologically similar to other parvovirus particles (Fig. 3B). In addition, three expected VP protein bands were detected by Western blotting in rAAV-GFP-infected cells, but not in the mock-infected cells (Fig. 3C).

3.3. Significantly more rAAAV is obtained by triple infection of insect cells

To compare the volumetric rAAAV yields of the two different production systems, the same number (2×10^8) of Sf9 or HEK 293 cells was used to produce rAAAV-GFP by triple infection or triple transfection. The quantitative PCR analysis showed that up to 28,000 rAAAV particles could be produced by each Sf9 cell, which was almost 25-fold more than that produced by each HEK 293 cell (Table1).

3.4. Insect and mammalian cell-produced rAAAV has similar gene transfer efficiencies for avian cells

To compare the gene transfer efficiencies of rAAAV produced by two different production systems, DF1 and CEL cells were infected with the same dose of rAAAV-GFP produced by Sf9 or HEK 293 cells. Fluorescent microscopy showed that the two types of avian cells could be transduced by rAAAV-GFP produced by Sf9 cells or HEK 293 cells with similar transduction efficiencies (Fig. 4).

4. Discussion

AAAV can be used as the efficient gene transfer vectors for avian cells. Although rAAAV vectors have been produced successfully by triple transfection of HEK 293 cells (Perozo et al., 2008; Wang et al., 2008; Wang et al., 2009), both scalability and viral titer of the transient transfection method can not meet the demand for in vivo experiments. Among the scalable rAAV production systems developed, insect cell/baculovirus expression system is the most promising platform for large-scale production of rAAV vectors (Galibert and Merten, 2011). This warranted us to explore the feasibility of insect cell/baculovirus expression system for scalable production of rAAAV vectors. To this end, we generated three recombinant baculoviruses containing AAV Rep genes, VP gene or ITR-flanked GFP reporter gene. Among these recombinant baculoviruses, the proportional expression of three VP

structural proteins is essential for the assembly of rAAV particles in insect cells. For example, co-infection of insect cells with three baculoviruses expressing AAV VP1, VP2 and VP3 individually leads to the formation of protein aggregates rather than correct rAAV particles (Urabe et al., 2002). The main reason is due to the presence of introns in the VP gene which can not be spliced out from the pre-mRNA by insect cells (Chen, 2008). This problem has been solved successfully by expressing the three VP proteins with mutated initiation codons in the absence of RNA splicing (Urabe et al., 2002). Therefore, in this study the ATG initiation codon of AAV VP1 was mutated to ACG to reduce the translation efficiency. The downstream out-of frame ATG codon with diminished translation of VP2 and VP3 was also modified to create an ACG triplet. In addition, the splice acceptor site downstream of the VP1 initiation codon was mutated to prevent the pre-mRNA splicing. Western blotting analysis of BacAVP-infected Sf9 cells showed that the three AAV VP proteins were expressed with the expected ratio, indicating the usability of the rBac for producing rAAV in insect cells.

To demonstrate the feasibility of above strategy for scalable production of rAAV vectors, insect cells were co-infected with BacARep, BacAVP and BacAGFP, and the infected cells were harvested for ultrathin section preparation and rAAV purification. Electron microscopy showed the presence of typical parvovirus particles clustered in the nucleus of rBac-infected cells. The rAAV particles could be purified to a relatively high purity by PEG precipitation. Western blotting analysis showed that the purified viral particles had a VP protein ratio similar to that of AAV. These data suggest the efficient expression of AAV proteins and correct assembly of the viral particles in the rBac-infected insect cells without the helper viruses. To compare the production performance of triple infection method with that of triple transfection, the same numbers of insect cells and HEK293 cells were used to produce GFP gene-containing rAAV vector. Quantitative PCR showed that each insect cell could produce 28,

000 rAAAV particles, which was almost 25-fold more than that (1100) of each HEK293 cell. Up to 5.6×10^{12} rAAAV could be obtained from 2×10^8 insect cells, indicating the relatively high scalability of insect cell/baculovirus expression system for producing rAAAV vectors.

Another important issue to be addressed was whether the rAAAV vector produced in insect cells could transfer gene of interest into avian cells. To answer this question, DF1 cells and CEL cells were transduced with the BacAGFP produced in insect cells or HEK293 cells under the same conditions. Fluorescent microscopy revealed the similar positive cell numbers in the two types of avian cell cultures after transduction with the rAAAV produced using two different methods. The expression of GFP reporter gene lasted for at least 2 weeks. These data suggest the potential use of insect cell-produced rAAAV vectors as efficient gene transfer vehicle for avian cells.

In conclusion, the insect cell/baculovirus expression system established in this study could become a scalable platform for producing rAAAV vectors, and the rAAAV produced could be used as efficient gene transfer vector for avian cells.

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293

Figure captions

Fig.1. The Schematic structures of recombinant baculovirus vectors used for producing rAAAV.

pFBARep harbors Rep78 and Rep52 expression cassettes. Rep78 is expressed by the p10 promoter and Rep52 is expressed by the ph promoter. pFBABVP expresses capsid proteins VP1, VP2, and VP3 under the control of ph promoter. The ATG codon of VP1 is mutated to ACG, enabling the expression of all three VP proteins from one transcript. pFBAGFP carries a GFP expression cassette flanked by AAV ITRs. GFP is expressed by the CMV promoter.

Fig.2. Detection of AAV Rep and Cap gene expression in the rBac-infected insect cells.

(A) Indirect immunofluorescence assay of AAV Rep and Cap gene expression in rBac-infected Sf9 cells using mouse anti-ARep or anti-AVP serum. (B) Western blotting assay of AAV Rep gene expression in rBacARep-infected Sf9 cells using mouse anti-ARep. (C) Western blotting assay of AAV Cap gene expression in rBacAVP-infected Sf9 cells using mouse anti-AVP. Lane M, prestained protein molecular weight marker; Lane 1, Sf9 cells not infected with recombinant baculovirus; Lane 2, Sf9 cells infected with recombinant baculovirus rBacARep or rBacAVP.

Fig.3. Identification of rAAAV-GFP produced in rBac-infected insect cells.

(A) Ultrathin section electron microscopy of Sf9 cells infected with the three recombinant baculoviruses, rBacAGFP, rBacAVP, and rBacARep. Original magnification: $\times 21\,000$. Bar: 100 nm. (B) Electron microscopy of purified rAAAV strained with 2% phosphotungstic acid. Original magnification: $\times 97\,000$. Bar: 100 nm. (C) Western blotting analysis of purified rAAAV. Lane 1, Sf9 cells not infected with recombinant baculovirus; Lane 2, purified rAAAV particles by PEG precipitation.

Fig.4. Detection of rAAAV-mediated GFP reporter gene expression in avian cells.

316 DF1 and CEL cells were infected with the same dose of rAAAV-GFP produced by Sf9 or HEK

317 293 cells and observed directly under microscope 24 h post-infection.

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Fig.1. (A) Transcription representations of the recombinant baculoviruses. rBacRep harbors Rep78 and Rep52 expression cassettes. Rep78 is expressed by the p10 promoter and Rep52 is expressed by the ph promoter. rBacVP expresses capsid proteins VP1, VP2, and VP3 under the control of ph promoter. The ATG codon of VP1 is mutated to ACG, enabling the expression of all three VP proteins from one transcript. rBacGFP carries a GFP expression cassette flanked by AAV ITRs. GFP is expressed by the CMV promoter. (B) Indirect immunofluorescence assay of recombinant proteins expressing in Sf9 cells infected with recombinant baculovirus. (C) Western blot analysis of recombinant proteins expressing in Sf9 cells infected with recombinant baculovirus. Lane 1 represents Sf9 cells infected with recombinant baculovirus rBacRep or rBacVP; Lane 2 represents Sf9 cells not infected with recombinant baculovirus.

Fig.2. Identification of rAAAV produced in Sf9 cells. (A) Ultrathin section electron microscopy of Sf9 cells infected with the three recombinant baculoviruses, rBacGFP, rBacVP, and rBacRep. Original magnification: $\times 21\,000$. Bar: 100 nm. (B) Electron microscopy of purified rAAAV strained with 2% phosphotungstic acid. Original magnification: $\times 59\,000$. Bar: 200 nm. (C) Western blotting analysis of purified rAAAV. Lane 1 represents Sf9 cells not infected with recombinant baculovirus; Lane 2 represents purified rAAAV.

Table 1. Comparison of rAAAV yield between two methods

	Total yield	Per cell	
	($\times 10^{11}$ VG)	($\times 10^3$ VG)	VG/TU ^a
Sf9 cells (2×10^8)	56 $\pm$ 1.6	28 $\pm$ 0.8	1562 $\pm$ 358
293 cells (2×10^8)	2.2 $\pm$ 0.89	1.1 $\pm$ 0.45	1936 $\pm$ 517

^aVector genomes per transducing unit.

Fig.3. Expression of GFP in DF1 cells or CEL cells infected with rAAV-GFP produced in Sf9 cells or 293 cells.