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Short Communication

Hybrid baculovirus–adeno-associated virus vectors for prolonged transgene expression in human neural cells

Chaoyang Wang

Institute of Bioengineering and Nanotechnology, Singapore

To prolong the transient gene expression mediated by baculoviral vector, the inverted terminal repeats and Rep gene of adeno-associated virus were incorporated into the genome of baculovirus, creating a hybrid baculovirus–adeno-associated viral (AAV) vector. By using previously constructed composite neuron-specific and astrocyte-specific promoters in this hybrid viral vector, sustained transgene expressions could be achieved in human neuronal and glial cell lines, but not in the correspondent rodent cell lines. This hybrid baculovirus-AAV vector might be useful for gene therapy of chronic neurological diseases in the central nervous system, such as Parkinson's disease and Alzheimer's disease. *Journal of NeuroVirology* (2008) **14,** 563–568.

Keywords: adeno-associated virus; baculovirus; gene therapy; hybrid viral vector

Introduction

Gene therapy has been considered as a potential approach to treat many diseases, including some neurological disorders, such as Parkinson's disease (PD) and Alzheimer's disease (AD) (Glorioso *et al,* 2003). Gene therapies of these chronic diseases, however, usually require long-term expressions of the delivered therapeutic genes, which largely depended on what vectors are used to deliver the genes. Among currently used viral vectors, adeno-associated virus (AAV) is the most commonly used one to drive long-term transgene expression by integrating its genome into the human chromosome 19 with the function of its inverted terminal repeats (ITRs) and Rep gene, and has been applied on clinical trials for gene therapy of PD and AD (Coura Rdos and Nardi, 2007). However, its inherited disadvantages like small capacity and inconvenience to prepare large scale of viral stock may hurdle its further clinical applications (Grieger and Samulski, 2005). Baculovirus is a recently developed gene delivery vector, which could efficiently transfer and express target genes in mammalian cells, with high transduction efficiency comparable to that of adenovirus (Sarkis *et al*, 2000; Ghosh *et al*, 2002). The advantages of baculovirus vector include large cloning capacity, ease preparation of virus stock with high titer, and lack of obvious pathogenicity, whereas the main limitation is its short transgene expression period (Kost and Condreay, 2002).

It would be highly desirable for gene therapy of chronic disease if we can combine the advantages of AAV and baculovirus together to create a hybrid baculovirus-AAV vector, which can mediate long-term gene expression with the integration function of AAV, while still remain the features of baculovirus of large cloning capacity and be easy to be prepared (Palombo *et al*, 1998). Because the ITRs and the Rep gene are the two elements of AAV that are responsible for the site-specific integration (McCarty *et al*, 2004), they can be incorporated into the backbone of the baculovirus genome to create a hybrid viral vector that can mediate long-term gene expression in human cells. Following this strategy, I constructed a hybrid baculovirus-AAV

Address correspondence to Dr. Chaoyang Wang at his current address: Genome Institute of Singapore, 60 Biopolis Street, #02-01, Genome, Singapore 138672. E-mail: wangc@gis.a-star.edu.sg

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vector in this study by flanking the gene-expressing cassette in baculovirus vector with the ITRs from AAV, and inserted the Rep gene into the baculovirus genome outside the ITRs flanking region.

Because there are many cell types in the central nervous system (CNS), cell type–specific gene expression is also very critical to gene therapy of CNS diseases (Beritez and Segovia, 2003; Deglon and Hantraye, 2005). To achieve long-term and cell type–specific gene expression in the CNS with the hybrid baculovirus-AAV vector, previously constructed neuron-specific CMV enhancer/platelet-derived growth factor (CMV E/PDGF) promoter and astrocyte-specific CMV enhancer/glial fibrillary acidic protein (CMV E/GFAP) promoter were used in the hybrid viral vector to drive long-term gene expression specifically in neurons and glial cells, respectively (Liu *et al*, 2004; Wang and Wang, 2006). The transgene expressions mediated by these hybrid viral vectors were examined *in vitro* with neuronal and glial cell lines. The results showed that prolonged transgene expressions can be achieved in human neuronal and glial cell lines, but not in correspondent rodent cells. This hybrid baculovirus-AAV vector, plus the composite cell type–specific promoters, might be useful for gene therapy of chronicle diseases in the CNS, such as PD and AD.

Results

The hybrid baculovirus-AAV vector with the CMV E/PDGF promoter driving luciferase gene was constructed and named HyV-CMV E/PDGF, in which the expression cassette was flanked by ITRs, and a Rep gene was inserted outside the ITRs flanked region. For comparison, baculovirus vector containing the ITRs but not the Rep gene and natural baculovirus vector with the same expression cassette were produced in parallel and named as BV-CMV E/PDGF-ITR and BV-CMV E/PDGF, respectively (Figure 1). Transgene expressions from these vectors were examined in neuronal cell lines HCN-2 (human) and PC12 (rat) at MOI (multiplicity of infection) of 25. The luciferase expressions from the infected cells were measured 1, 3, 7, and 14 days post-infection. The results showed that, in human HCN-2 cells, a more sustained luciferase expression can be observed with the hybrid baculovirus-AAV vector that contains both the ITRs and Rep, as compared with the baculovirus vector containing ITRs only or natural baculovirus vector. At day 14, the luciferase activity in hybrid virus infected HCN-2 cells decreased to about 50% of that at day 1, whereas the corresponding percentages were less than 20% in HCN-2 cells infected with the baculovirus vector containing the ITRs only or the natural baculovirus vector (Figure 2, *top*). However, in PC12 cells, which originated from rat, no such results could be observed. The transgene expressions mediated by all the three viral vectors decreased at similar rates, although their expression levels were slightly different (Figure 2, *bottom*).

Hybrid baculovirus-AAV vectors containing the astrocyte-specific CMV E/GFAP promoter were also constructed and prepared (Figure 1). Glioma cell lines U251 (human) and C6 (rat) were infected with HyV-CMV E/GFAP, BV-CMV E/GFAP, and BV-CMV E/GFAP-ITR with MOI of 25, respectively, and the luciferase expressions were measured 1, 3, 7, and 14 days after the infection. Similarly, the results showed that a prolonged transgene expression can be achieved in human glioma cell lines with the hybrid baculovirus-AAV vector that contains both the ITRs and the Rep gene (Figure 3, *top*), but not in rat C6 cell lines (Figure 3, *bottom*).

Site-specific integration in human chromosome 19 AAVS1 site mediated by the hybrid baculovirus-AAV vector was then examined. Neuronal cells HCN-2 were infected with HyV-CMV E/PDGF and BV-CMV E/PDGF-ITR, respectively. Four days after the infection, genomic DNAs of the infected cells were extracted, and were used for the amplification of ITR-AAVS1 junction by nested polymerase chain reaction (PCR), in which two sets of primers specific for the luciferase gene and the AAVS1 of chromosome 19 were used, therefore, only site specifically integrated luciferase gene on the AAVS1 site can be amplified by the PCR reaction. As shown in Figure 4, specific DNA band with the expected size of about 0.8 kb was amplified from HCN-2 cells infected with the hybrid baculovirus-AAV vector, but not from cells infected with baculovirus vector with ITR only. The same PCR was done with rat PC12 cells that were also infected with HyV-CMV E/PDGF and BV-CMV E/PDGF-ITR, respectively. As expected, no specific DNA band was amplified from both of the infected PC12 cells because there is no AAVS1 site on rat chromosomes (data not shown).

Discussion

The application of baculovirus as a gene delivery vector for chronic neurological diseases might be hampered by its transient gene expression, whereas the obstacles for applying AAV vector for CNS gene delivery were its small capacity for foreign genes and inconvenience of viral preparation. The hybrid baculovirus-AAV vector overcomes the disadvantages of both baculovirus and AAV vector, making itself a promising vehicle for gene therapy of neurological disorders, especially for neurodegenerative diseases that require long-term expression of the therapeutic gene. In this study, hybrid baculovirus-AAV vectors carrying the neuron-specific CMV E/PDGF promoter or astrocyte-specific CMV

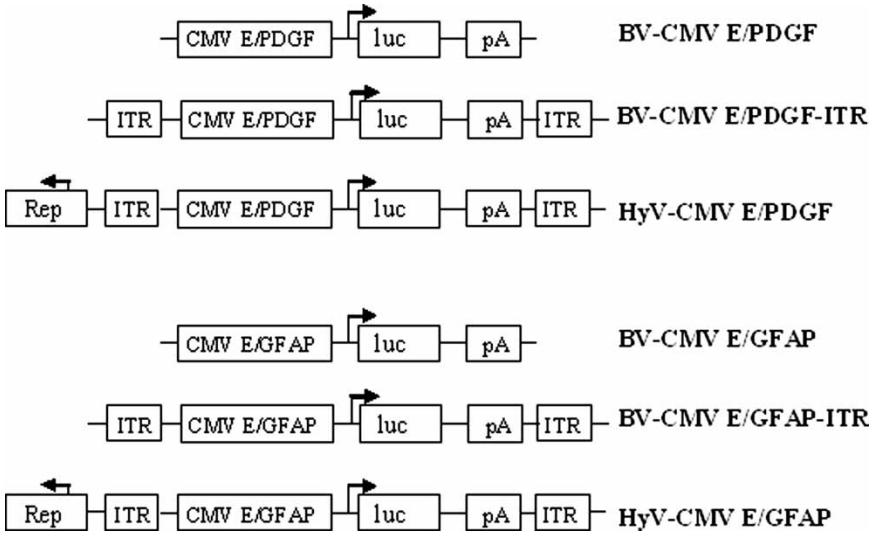

Figure 1 Expression cassettes of the hybrid baculovirus-AAV vector and baculovirus vectors used in this study. CMV E/PDGF, CMV enhancer/PDGF promoter; CMV E/GFAP, CMV enhancer/GFAP promoter; luc, luciferase gene; pA, polyA tail; ITR, inverted terminal repeats of AAV; Rep: the *Rep* gene of AAV.

E/GFAP promoter were produced and their *in vitro* gene delivery were examined with infection of human and nonhuman cell line. A sustained transgene expression could be observed in human originated neural cell line with the hybrid baculovirus-AAV vector, but not in the correspondent rodent cell line. ITRs and Rep gene are the two elements in AAV to mediate the integration of ITR-flanking gene into human chromosome at 19q13.4 qtr (AAVS1), which is unique for human chromosome, not the chromosome of mouse or rat (McCarty *et al*, 2004). This explains why we did not observe any prolonged transgene expression in rodent cell lines, and also implies that the prolonged gene expression in human cell lines observed in this study could be attributed to the site-specific integration, which is primarily proved by the nested PCR in this study, but might need to be further examined by fluorescent *in situ* hybridization (FISH) or chromosome PCR and sequencing.

Using promoters with cell type–specificities instead of viral promoters such as CMV promoter can restrict the transgene expression within the desire cells and therefore minimizing the side effects caused by transduction of unintended cells. In this study, composite neuron-specific or astrocyte-specific promoter with high transcriptional activity was employed by the hybrid baculovirus-AAV vector to achieve a high level, cell type–specific, and long-term transgene expression in the CNS. Although the *in vivo* transduction of this gene delivery system was not examined in this study, it can be anticipated that the cell type specificities of the hybrid CMV E/PDGF promoter and CMV E/GFAP promoter would be reserved in the hybrid baculovirus-AAV vector, as we tested them before in baculovirus or AAV vectors (Wang and Wang, 2005, 2006).

The hybrid viral vector developed in this study can be applied for gene therapy of neurodegenerative diseases in the CNS such as PD and AD, which usually require long-term and cell type–specific expressions of therapeutic genes. Targeted delivery of therapeutic genes like glial cell line–derived neurotrophic factor (GDNF) to neurons in substantia nigra can prevent the lose of dopaminergic neurons, therefore prevent the happening of PD (Carlsson *et al*, 2007). Astrocyte-specific expression of brain-derived neurotrophic factor (BDNF) or tyrosine hydroxylase has been used for therapy of PD, and transgene expression of apolipoprotein E in astrocytes was applied for AD (Segovia *et al*, 1998; Costantini *et al*, 2000). By using the hybrid baculovirus-AAV vector developed in this study, long-term and cell type–specific gene delivery into CNS can be achieved with conveniently prepared viral stocks, providing a useful gene delivery vehicle for gene therapy of chronicle diseases in the CNS. Because the long-term gene expression mediated by the hybrid viral vector can only be achieved in human cells, we only perform the *in vitro* studies. Necessary *in vivo* study can be carried out in the future with transgenic rats and mice that carry the AAVS1 3.5-kb DNA fragment for AAV-mediated site-specific integration (Rizzuto *et al*, 1999).

Materials and methods

Construction and preparation of the viral vectors
To construct the hybrid baculovirus-AAV vector, an expression cassette containing a MCS (multiple cloning site), a reporter gene encoding luciferase, a SV40 polyA signal, and two ITR sequences at both ends was amplified from pAAV plasmid, and

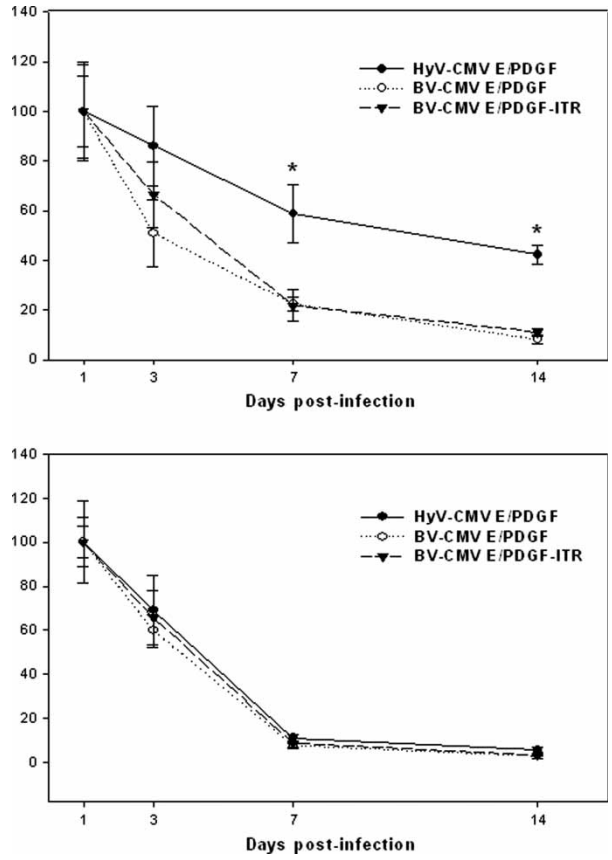

Figure 2 Transgene expressions in neuronal cells HCN-2 (*top*) and PC12 (*bottom*) mediated by viral vectors containing the CMV E/PDGF promoter. Cells were infected with HyV-CMV E/PDGF, BV-CMV E/PDGF, and BV-CMV E/PDGF-ITR at MOIs of 25, and the luciferase expressions were measured 1, 3, 7, and 14 days after the infection. The luciferase expression at each time point was presented as relative to the expression at day 1, which was set as 100 ($n = 4$). The asterisk (*) indicates these values of HyV-CMV E/PDGF are statistically significant ($P < .05$) in comparison to BV-CMV E/PDGF or BV-CMV E/PDGF-ITR.

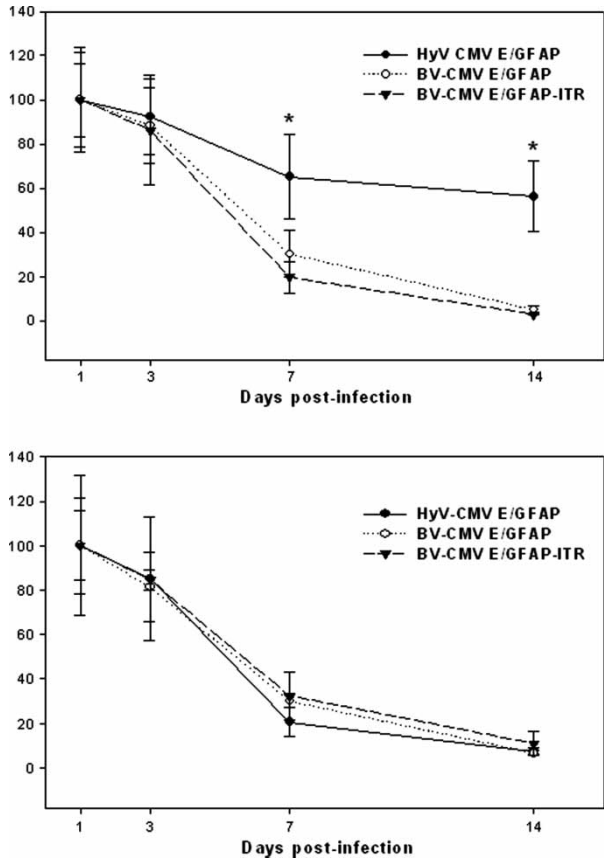

Figure 3 Transgene expressions in a human glioma cell line U251 (*top*) and a rat glioma cell line C6 (*bottom*) mediated by viral vectors containing CMV E/GFAP promoter. Cells were infected with HyV-CMV E/GFAP, BV-CMV E/GFAP, and BV-CMV E/GFAP-ITR at MOI of 25, and the luciferase expressions were measured 1, 3, 7, and 14 days after the infection. The luciferase expression at each time point was presented as relative to the expression at day 1 which was set as 100 ($n = 4$). The asterisk (*) indicates these values of HyV-CMV E/GFAP are statistically significant ($P < .05$) in comparison to BV-CMV E/GFAP or BV-CMV E/GFAP-ITR.

inserted into a baculovirus vector, pFB1 (Gibco BRL, USA), between Avr II and Sal I. A DNA fragment containing the full sequences of Rep gene was amplified from pSub201, which has been digested with Apa I to remove the encoding sequence of Cap gene and ligated again. The Rep gene was then digested with Rsr II and inserted into Rsr II site of pFB1 vector outside the ITRs. CMV E/PDGF promoter and CMV E/GFAP promoter were used to drive the luciferase reporter gene in the hybrid baculovirus-AAV vector (Liu *et al*, 2004; Wang and Wang, 2006). Unmodified baculovirus vectors and baculovirus vectors with ITRs but no Rep gene were also constructed with the same luciferase expression cassette driven by the CMV E/PDGF promoter and CMV E/GFAP promoter, respectively (Figure 1). All the viral vectors were produced, propagated, and purified according to the manufacturer's instructions (Gibco BRL, USA). The titers (PFU [plaque-forming units]) of the virus were determined by plaque assay on sf9 insect cells.

In vitro *viral infection and gene expression analysis*
For *in vitro* viral infection, neuronal cell lines HCN-2 (human) and PC12 (Mouse) and glioma cell lines U251 (human) and C6 (rat) were used. Cells were seeded in 48-well plates with densities of 20,000 cells/well. After the attachment of the cells, appropriate amounts of hybrid baculovirus-AAV vectors or baculovirus vectors were added in 100 μl of serum-free Dulbecco's modified Eagle's medium (DMEM), and incubated with the cells at

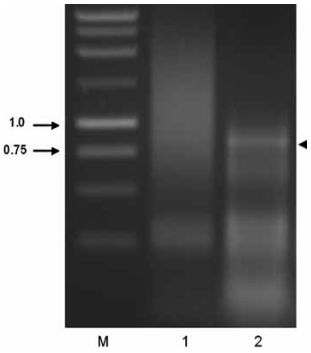

Figure 4 Site-specific integration mediated by hybrid baculovirus-AAV vectors in human cell line. HCN-2 cells were infected with HyV-CMV E/PDGF and BV-CMV E/PDGF-ITR, respectively. Four days after the infection, genomic DNAs were extracted and nested PCR were carried out to amplify the ITR-AAVS1 junction. 1: BV-CMV E/PDGF-ITR; 2: HyV-CMV E/GFAP; M: DNA marker. The amplified band with about 800 bp is indicated with arrows. The molecular size standards (kb) are indicated.

37°C for 1 h. After the incubation, serum-free DMEM containing the viruses was replaced by fresh growth medium, and the cells were continued to incubate at 37°C for appropriate time before analysis. For luciferase assay, cells were washed with phosphate-buffered saline (PBS) and permeabilized with 100 μl of reporter cell lysis buffer (Promega, WI, USA). The total protein concentration of each lysate was determined by using the DC Protein Assay (Bio-Rad). Ten microliters of cell extract was used for luciferase assay with a luciferase assay kit (Promega) in a single-tube luminometer (Berthold Lumat LB 9507, Bad Wildbad, Germany). The luciferase expressions were expressed in relative light units (RLU) per milligram of total protein. For the comparison of results from different groups, the luciferase activity at day 1 in each group was set as 100.

Nested PCR

Integration of ITR-flanked DNA in the AAVS1 site of chromosome 19 was determined by nested PCR with primer pairs that flank the AAV-chromosome junction. Primers Cr1 and Cr2 were located on the chromosome 19 around the AAVS1 site, whereas primers Luc1 and Luc2 were located within the luciferase gene that carried by the vectors. Cr1 and Luc1 were used for the first round of PCR amplification with 0.5 μg of genomic DNA that was extracted from cells infected by the hybrid viral vectors. The PCR amplification was carried out with initial incubation at 94°C for 4 min, followed by 30 cycles of amplification with 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. One percent of the amplification product was diluted into a new PCR reaction using the Cr2 and Luc2 primers with the same PCR parameters. The product of second round PCR was analyzed on 1% agarose gel. The sequences of the oligo primers were as follow:

Cr1: 5′-GCGCGCATAAGCCAGTAGAGC
Cr2: 5′-ACAATGGCCAGGGCCAGGCAG
Luc1: 5′-CGGGCGCGGTCGGTAAAG
Luc2: 5′-TGGGCGTTAATCAAAGAG

Declaration of interest: The author reports no conflicts of interest. The author alone is responsible for the content and writing of the paper.

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