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Successful Transduction of Mammalian Astrocytes and Oligodendrocytes by "Pseudotyped" Baculovirus Vector *in Vitro* and *in Vivo*

Hiroshi KOBAYASHI[1,2], Kazuhiko WATABE[3], Sayoko IZUKA[1], Hideki TANI[4], Yoshiharu MATSUURA[4], James BARSOUM[5], Campbell KAYNOR[5], Toya OHASHI[1,2], and Yoshikatsu ETOH[1,2]

[1]*Department of Gene Therapy, Institute of DNA Medicine, The Jikei University School of Medicine*
[2]*Department of Pediatrics, The Jikei University School of Medicine*
[3]*Department of Molecular Neuropathology, Tokyo Metropolitan Institute for Neuroscience*
[4]*Research Center for Emerging Infectious Diseases, Research Institute for Microbial Diseases, Osaka University*
[5]*Biogen, Inc.*

ABSTRACT

Baculovirus vectors can efficiently transduce human hepatoma cells and primary hepatocytes in culture. We report the potential use of baculovirus as a vector for gene delivery into cells of the mammalian central nervous system. We generated a "pseudotyped" baculovirus encoding the bacterial β-galactosidase (β-Gal) gene (LacZ), under the control of the cytomegalovirus promoter, and the vesicular stomatitis virus G protein gene, under the control of the polyhedrin promoter. This virus was used to infect primary cultures of rat glial cells. Three days after infection, these cells were immunostained for β-Gal, glial fibrillary acidic protein (for astrocytes), or galactocerebroside (for oligodendrocytes) to identify the infected cell types. Positive β-Gal immunofluorescence was observed in 10.4% of glial fibrillary acidic protein-positive cells and 35.6% of galactocerebroside-positive cells at a multiplicity of infection of 50. When the virus was injected into adult mouse striatum, β-Gal-positive cells were demonstrated, and no cytological or histological evidence of cell damage, inflammation, or cell infiltration was observed after infection. These findings suggest that baculovirus-mediated gene transfer can be used for gene therapy against nervous system diseases, especially demyelinating disorders, affecting mainly oligodendrocytes.

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Key words : baculovirus, gene therapy, pseudotype, astrocytes, oligodendrocytes

INTRODUCTION

The baculovirus vector has been widely used to obtain high levels of expression of foreign genes under the control of the strong baculoviral promoter (polyhedrin promoter) in insect cells[1]. Although its host specificity had been thought to be restricted to insect cells, the recombinant baculovirus was recently shown to be capable of transferring and expressing foreign genes in mammalian cells, such as hepatocytes[2,3] and nonhepatic cell lines[4]. For the original baculovirus vector the foreign gene is cloned next to the viral polyhedrin promoter, whereas for the recombinant baculovirus the gene is fused to a mammalian pro-

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小林 博司，渡部 和彦，飯塚佐代子，谷 英樹，松浦 善治，James Barsoum, Campbell Kaynor, 大橋 十也，衛藤 義勝
Mailing address : Hiroshi KOBAYASHI, Department of Gene Therapy, Institute of DNA Medicine, The Jikei University School of Medicine, 3-5-28, Nishi-Shimbashi, Minato-ku, Tokyo 105-8461, Japan.
E-mail address : hr-kb@wd6.so-net.ne.jp

moter, such as cytomegalovirus immediate early promoter and inserted. Efficient gene transfer has been reported, and no viral DNA replication has been observed in mammalian cells. These results suggest that this virus system would be safe for humans.

Sarkis et al. have reported that the baculovirus vector can be used to transfer a marker gene to neurons and astrocytes *in vitro* and *in vivo*[5]. They have reported efficient gene transfer into astrocytes and neurons, but not into oligodendrocytes. Oligodendrocytes are the cells mainly affected in several neurological diseases, such as demyelinating disorders; therefore, for gene therapy in these diseases, investigating ways to transfer genes into oligodendrocytes is important. Barsoum et al. have reported that pseudotype baculovirus, expressing vesiculostomatitis virus G (VSVG) protein in its envelope, transduces mammalian cells much more efficiently than does nonpseudotype baculovirus[6]. In this study, we examined the ability of a pseudotype baculovirus vector to transfer a marker gene in central nervous system (CNS) cells, including oligodendrocytes.

MATERIAL AND METHODS

1. *Construction of pseudotype transfer plasmids*

The pseudotype baculovirus transfer vector, pCZPG (Fig. 1), was generated by inserting expression cassettes encoding the VSVG protein and bacterial β-galactosidase (β-Gal) gene (LacZ) into the standard baculovirus transfer vector BacPak9 (Clontech Laboratories, Inc., Palo Alto, CA, USA)[6]. First, the VSVG gene complementary DNA was excised from the plasmid pLGRNL[7]. This fragment was inserted into the *Bam*HI site of BacPak9, in a direct orientation with respect to the polyhedrin promoter, to create VSVG/BP9. Next, the LacZ gene preceded by the cytomegalovirus promoter and followed by the SV40 poly(A) signal was inserted into VSVG/BP9 such that the LacZ cassette was downstream from the VSVG gene and the direction of transcription was convergent[6].

2. *Pseudotype baculovirus production*

Cells of the insect cell line Sf9 were grown in TC-100 medium (GIBCO/Invitrogen, Carlsbad, CA, USA) with 0.26% Bacto tryptose phosphate broth (Difco, Detroit, MI, USA), 100 μg/ml kanamycin, and 10% fetal bovine serum (GIBCO/Invitrogen). Recombinant pseudotype baculovirus, CZPG, was generated by homologous recombination, as described previously[4,9]. Briefly, pCZPG and the *Bsu* 36I-digested baculovirus genomic DNA were cotransfected into Sf9 cells by lipofectin (GIBCO/Invitrogen). Two days later, the culture medium was harvested and used to infect Sf9 cells in a standard plaque assay. The plaques were isolated and purified by a second round of plaque isolation. After the presence of the predicted recombinant DNA restriction digestion pattern was determined, selected plaques were expanded twice in tissue culture flasks and then on a large scale in 100-ml spinner cultures to obtain a significant volume of virus preparation. The viral titer was determined with a plaque assay[10]. To purify the virus, conditioned media of Sf9 cells infected with the virus were harvested 3 days after infection, and cell debris was removed by centrifugation at 6,000 g for 15

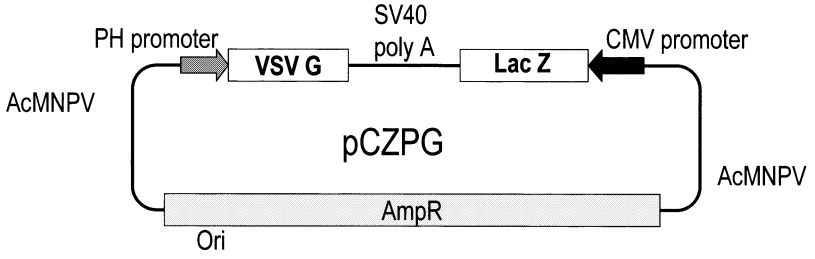

Fig. 1. Structure of pseudotype baculovirus transfer plasmid (pCZPG). AcNMPV, Baculovirus sequences; PH, polyhedrin promoter; AmpR, ampicillin-registance gene; Ori, replication origin; VSVG, Vesicular stomataitis virus G protein gene.

minutes at 4℃. The virus was pelleted by ultracentrifugation at 80,000 g (RPS27-2 rotor, Hitachi, Tokyo) for 90 minutes and resuspended in 1 ml of phosphate buffered saline (PBS), loaded on 10% to 60% (w/v) sucrose gradients, and ultracentrifuged at 77,600 g (P-40ST rotor, Hitachi) for 90 minutes. The virus band was collected and resuspended in PBS and ultracentrifuged at 77,600 for 90 minutes. The virus pellet was resuspended in PBS, and infectious titers were determined with a plaque assay[4].

3. Mixed glial cell culture

Cultures of rat astrocytes and oligodendrocytes were established with the enzyme digestion-Percoll (Pharmacia, Uppsala, Sweden) density gradient method[11-13]. The brains of 10-week-old male Wistar rats were minced and incubated in 0.25% trypsin and 20 μg/ml DNase in calcium- and magnesium-free Hanks balanced salt solution (HBSS) for 40 minutes at 37℃. Dissociated cells were passed through a 100-μm nylon mesh. Isolated cells suspended in HBSS were mixed with Percoll, and a gradient was formed by centrifugation for 25 minutes at 15,000 g in a high-speed refrigerated centrifuge with a fixed-angle rotor (Hitachi). The final concentration of Percoll was 30% in HBSS. An astrocyte- and oligodendrocyte-enriched fraction, bound by an upper myelin layer and a lower erythrocyte layer, was collected and diluted in three volumes of HBSS, before being harvesting by low-speed centrifugation for 10 minutes. The cells were washed twice in HBSS, suspended in feeding medium (1×10^5 cells/ml), and seeded on polysine-coated 9-mm-diameter round coverslips (Aclar, Honeywell/AlliedSignal, Pottsville, PA, USA). The feeding medium consisted of 5% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin in Isocove's modified Dulbecco's minimum essential medium. The cultures were maintained in 5% CO_2 at 37℃ for 2 weeks, with the culture medium being changed every 4 days.

4. Infection of glial cell cultures with CZPG

Living cells of mixed rat glial cultures on coverslips after 2 weeks in vitro were infected with a 10-μl solution of purified CZPG at a multiplicity of infection of 50 for 1 hour and were incubated in feeding medium for 3 days in 5% CO_2 at 37℃. To detect β-Gal expression in infected cells, immunofluorescence was performed with a rabbit antibody to β-Gal. Cell types were identified with immunofluorescence using antibodies to glial fibrillary acidic protein (GFAP) and galactocerebroside (GC), specific markers for astrocytes and oligodendrocytes, respectively. For double immunofluorescence staining for β-Gal and GFAP, cells on coverslips were fixed in 1% paraformaldehyde in PBS for 10 minutes at 4℃ and in methanol for 10 minutes at −20℃ and incubated at room temperature for 1 hour with a mixture of a rabbit antibody to β-Gal (Eppendorf-5 Prime, Boulder, CO, USA) and rat monoclonal antibody (hybridoma soup) to GFAP[14] (kindly provided by Dr. Seung U. Kim, University of British Columbia) at final dilutions of 1 : 50 and 1 : 2, respectively. This incubation was followed by incubation at room temperature for 1 hour with a mixture of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Cappel, MP Biomedicals, Aurora, OH, USA) and rhodamine-conjugated goat anti-rat IgG (Cappel) at a final dilution of 1 : 50. For double immunofluorescence staining for β-Gal and GC, living cells on coverslips were incubated with mouse monoclonal antibody (hybridoma soup) to GC[15] (kindly provided by Dr. Seung U. Kim) at a dilution of 1 : 2 for 30 minutes at room temperature. After being washed with Isocove's modified Dulbecco's minimum essential medium, the cells were incubated with rhodamine-conjugated goat anti-mouse IgG (Cappel) at a final dilution of 1 : 50 for 30 minutes at room temperature. The cells were fixed with 1% paraformaldehyde in PBS at 4℃ for 10 minutes and cold methanol at −20℃ for 10 minutes. After being washed in PBS, the cells were incubated with rabbit antibody to β-Gal at a dilution of 1 : 50 for 1 hour at room temperature. After being washed, the cells were incubated with FITC-conjugated goat anti-rabbit IgG at a final dilution of 1 : 50 for 45 minutes at room temperature. After being washed, coverslips were mounted on glass slides with 20% glycerol/10% polyvinylalcohol in 0.1 M Tris-HCl buffer, pH 8.0. Cells were then examined under a universal microscope (Olympus Opti-

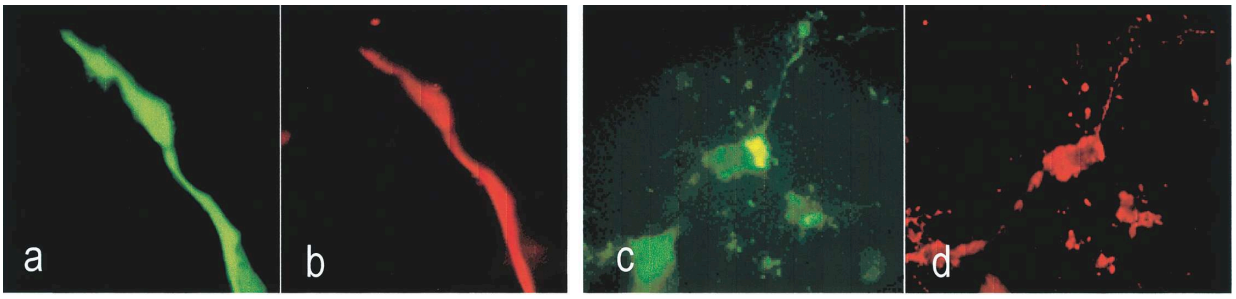

Fig. 2. β-Gal expression in rat primary culture astrocytes and oligodendrocytes *in vitro*. a, b: Astrocytes infected with CZPG *in vitro* for 3 days. β-Gal-FITC (a) and GFAP- rhodamine (b) double immunofluorescence. c, d: Oligodendrocytes infected with CZPG *in vitro* for 3 days. β-Gal-FITC (c) and GC-rhodamine (d) double immunofluorescence.

cal Co., Tokyo) equipped with fluorescein and rhodamine optics.

5. Infection of mouse brains with CZPG

Intracerebral injection of purified CZPG was carried out as follows: 6- to 8-week-old normal female C57BL/6 mice ($n=12$) were anesthetized with intraperitoneal injection of 40 mg/kg of pentobarbital sodium. Five microliters of purified CZPG (1×10^9 pfu/ml) was injected slowly into the left striatum (AP: to 1 mm; ML: 2 mm; DV: 3.6 mm to the bregma) or the ventricle (AP: to 1 mm; ML: 2 mm; DV: 2.6 mm to the bregma) through a syringe (Hamilton Co., Reno, NV, USA). Three days after the operation, the mice were deeply anesthetized through inhalation of diethyl ether and intracardially perfused with 4% paraformaldehyde in PBS. The brains were removed, postfixed in the same fixative for 24 hours, and cryoprotected in 30% sucrose in PBS, after which 20-μm-thick coronal sections were cut with a cryostat. Gene transfer into the CNS was identified with β-Gal staining. These sections were washed four times in PBS and stained in a solution of 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 2 mM $MgCl_2$ in PBS[16]. Cell types were identified with immunofluorescence using antibodies to GFAP and 3′,5′-cyclic nucleotide phosphodiesterase (CNP) for astrocytes and oligodendrocytes, respectively. The sections were washed in PBS and incubated in 0.1% Triton X-100 in PBS (PBST) for 30 minutes and in 3% normal goat serum in PBST for 1 hour at room temperature. For double immunofluorescence staining for β-Gal and GFAP, the sections were incubated at 4°C overnight with a mixture of rabbit antibody to β-Gal and rat monoclonal antibody to GFAP at final dilutions of 1:50 and 1:2, respectively. This incubation was followed by incubation at room temperature for 1 hour with a mixture of FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-rat IgG at a final dilution of 1:50. For double immunofluorescence staining for β-Gal and CNP, the sections were incubated at 4°C for 12 hours with a mixture of rabbit antibody to β-Gal and mouse monoclonal antibody to CNP at final dilutions of 1:50 and 1:100, respectively. This incubation was followed by incubation at room temperature for 1 hour with a mixture of FITC-conjugated anti-rabbit IgG and rhodamine-conjugated anti-mouse IgG at a final dilution of 1:50.

RESULTS

1. Transduction of cultured glial cells by CZPG

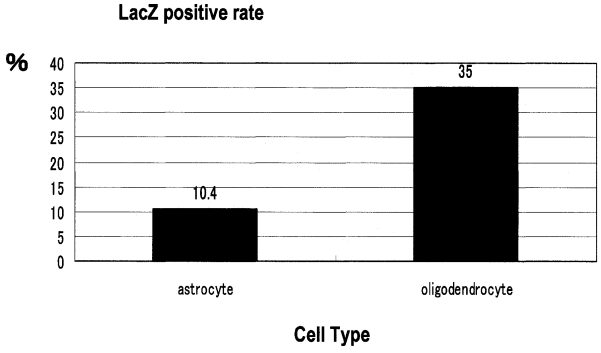

Fig. 3. Efficiency of LacZ gene transfer into rat primary culture *in vitro*.

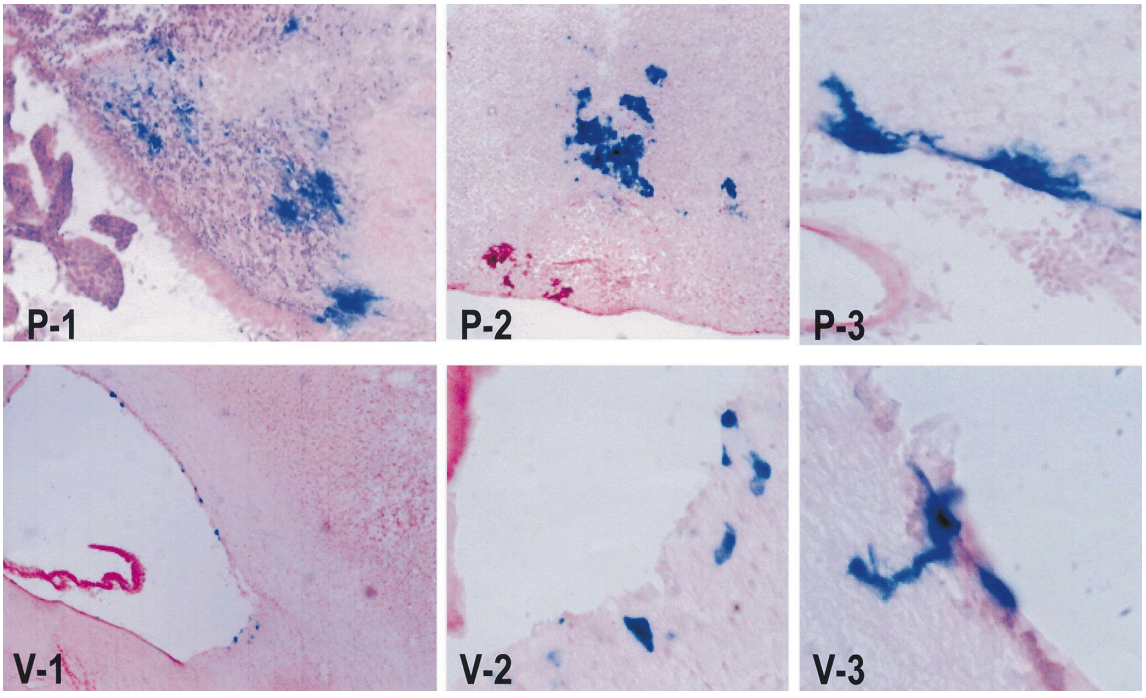

Fig. 4. β-Gal expression in normal mouse brain *in vivo*. 3 days after injection of CZPG into normal mouse (C57BL/6) striatum, gene transfer into CNS *in vivo* was identified by β-Gal staining.
P1-P3 ; the slides of the mouse brain injected in the parenchyma. Lac Z positive cells were seen in near the choroid plexus (P-1), striatum (P-2), and corpus callosum (P-3).
V1-V3 ; the slides of the mouse brain injected in the ventricle. Lac Z positive cells were seen in the ependimal cell (V-1, 2) and partially migrated into the parenchyma (V-3).

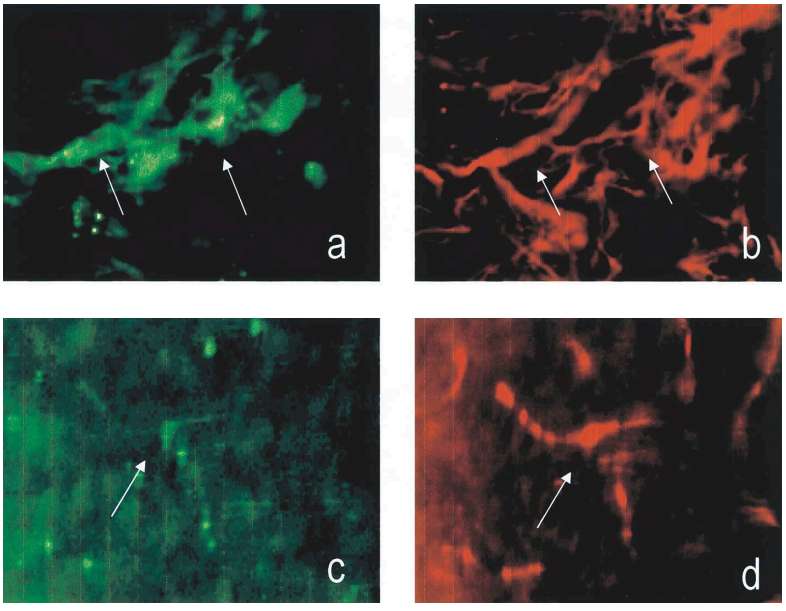

Fig. 5. The slides of the Immunohistochemistry of the mouse brain injected in the parenchyma, double-positive cells, i.e. β-Gal+/GFAP+ (an infected astrocytes ; a, b) and β-Gal+/CNP+ (infected oligodendrocyte : c, d) cells were demonstrated by double immunofluorescence microscopy, indicating both astrocytes and oligodendrocytes can also be transduced *in vivo* by CZPG.

Under immunofluorescence microscopy, mixed glial cell cultures infected with CZPG for 3 days showed double-positive cells, i.e., β-Gal+/GFAP+ cells (infected astrocyte) and β-Gal+/GC+ cells (infected oligodendrocytes), indicating that both astrocytes and oligodendrocytes were successfully transduced to express β-Gal encoded by CZPG (Fig. 2). Of 192 GFAP+cells, 20 were positive for β-Gal (transduction efficiency; 10.4%). Of the 202 GC+ cells, 72 (35%) were positive for β-Gal. These results indicate that CZPG can transduce oligodendrocytes more efficiently than astrocytes in primary culture of adult rat brain (Fig. 3).

2. Efficient transduction of neuronal cells in vivo by CZPG

To test the ability of CZPG to infect glial cells in vivo, we injected CZPG into the mouse striatum (n=12). Three days after injection, β-Gal positive cells were demonstrated in the parenchyma (Fig. 4 P1, P2, P3) and periventricular zone (Fig. 4 V1, V2, V3). We detected transduction mainly for parenchymal cells (P1, P2, P3) and ependymal cells (V1, V2), partially migrated into the parenchyma (V3). Next, we tried immunohistochemistry *in vivo*, double-positive cells, i.e., β-Gal+/GFAP+ cells (infected astrocytes) and β-Gal+/CNP+ cells (infected oligodendrocytes) were demonstrated by double immunofluorescence microscopy, indicating that both astrocytes and oligodendrocytes can also be transduced *in vivo* by CZPG (Fig. 5). We observed no cytological damage *in vitro* and no histological evidence of cell damage, inflammation, or cell infiltration after infection with CZPG.

DISCUSSION

Gene therapy for neurological disorders requires an efficient and stable gene delivery system for the CNS. As for gene transfer systems into the CNS cells, adenovirus, adeno-associated virus (AAV), herpesvirus, and lentivirus vectors have been studied[17]. Although adenovirus vector has been reported to be a useful gene transfer system for the nervous system[18–20], its expression is transient because the vector does not integrate into the host genome. Moreover, first-generation adenovirus vectors cause severe tissue inflammation when inoculated in brain tissue[21]. The adeno-associated virus-mediated gene transfer system is promising because no cellular toxicity has been reported and long-term expression by integration has been reported[20,22,23]. However, a problem with this system is the difficulty of preparing a sufficiently large virus stock for clinical use. Herpes simplex virus-based vectors have particular advantages for gene delivery into the nervous system including their ability to infect nondividing neurons and long-term expression. Disadvantages of this system are host immune responses and inflammatory and toxic reactions[24,25]. Some investigators have reported that lentivirus vector is promising for the transfer of genes into CNS cells, although its safety remains unclear[26,27].

To seek an alternative approach to overcome the limitations of current vector systems for transducing CNS cells, we tested a baculovirusmediated gene transfer system in rodent CNS cells in vivo and in vitro. The generation of recombinant baculovirus is relatively less time-consuming, and expansion of the virus stock is relatively easy. The titer of the virus stock after purification is high and comparable to that of adenovirus. Large DNA (up to 15 kb) can be inserted into the transfer vector. Moreover, baculovirus cannot replicate in mammalian cells, and no cell toxicity has been observed[28]. In fact, there was no evidence of tissue inflammation or cell damage in the present study.

The first attempt to transfer genes to mammalian cells with the baculovirus vector was reported by Hofman et al. in 1995. They successfully transferred a gene to mammalian hepatocytes. However, they failed to transfer genes to neural cell lines, such as mouse neuroblastoma Neuro-2a and human astrocytoma SW 1088[2]. Recently, Sarkis et al. have reported successful transduction of nervous system cells (both neurons and astrocytes) *in vitro* and *in vivo* using nonpseudotype baculovirus[5]. In the present study, we used a pseudotype vector. By pseudotyping, the VSVG protein is expressed in the viral envelope and mediates the escape of the recom-

binant pseudotype baculovirus from the intracellular vesicles. The efficiency of the escape may be the rate-limiting step for transduction[6]. We also tried a nonpseudotype baculoviral vector and detected efficient transduction *in vitro*, especially in the liver cell line Hep G2, but detected no transduction *in vivo* (data not shown). The baculovirus vector CZPG prepared in this study could transduce both astrocytes and oligodendrocytes. This ability implies a potential advantage of pseudotype baculovirus vectors for use in CNS gene therapy, because oligodendrocytes are the cells principally affected in many neurological diseases, such as globoid cell leukodystrophy (Krabbe's disease), metachromatic leukodystrophy, and adrenoleukodystrophy. We are now generating a baculovirus vector expressing galactocerebrosidase, which is lacking in Krabbe's disease, to investigate its therapeutic effects in a mouse model of Krabbe's disease. Our failure to find cytological or histological evidence of cell damage, inflammation, or cell infiltration after infection of CZPG *in vitro* and *in vivo* point is also important for gene transfer into CNS and other organ systems *in vivo*.

There are several unanswered questions regarding our baculovirus vector system ; i.e., how long the expression persists and how efficiently it infects oligodendrocytes *in vivo*. For the treatment of Krabbe's disease, highly efficient transduction of oligodendrocytes and persistent expression of transferred genes will be required. Additional experiments are underway to answer these questions.

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