Published in final edited form as:

J Biotechnol. 2013 July 20; 166(4): 182–186. doi:10.1016/j.jbiotec.2013.05.011.

Evaluation of Cre Recombinase Delivery in Mammalian Cells using Baculovirus Infection

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Abstract

In vivo conditional knock-out of a protein is a method of choice to decipher its biological function. It can be achieved by encoding the cre-recombinase on a recombinant virus to exert spatio-temporal control of its expression and enzymatic activity and, subsequently, of the target gene deletion. Recombinant baculoviruses have been successfully used to express a wide range of proteins in insect cells. More recently, their potential to infect mammalian cells has been addressed but, so far, their ability to yield a conditional knock-out as a result of efficient *in vivo* cre-recombinase gene delivery has not been examined. Cre-recombinase fused to the green fluorescent protein was cloned under the control of the CAG promoter in a recombinant *Autographa californica* baculovirus expressing the vesicular stomatitis virus envelope G protein for increased mammalian cell infection. Gene delivery was evaluated *in vitro* in mammalian cells, neuroblastoma and mouse primary neuronal cultures as well as *in vivo* in the mouse brain. Infection with adeno-associated viruses encoding the cre-recombinase fused to the green fluorescent protein was performed as a positive control. Our results indicate that baculovirus infection leads to functional cre-recombinase expression in non-neuronal and neuroblastoma cell lines but not in mouse primary neuronal cultures or brain.

Keywords

Baculovirus; cre recombinase; adeno associated virus; mammalian cell; mouse brain

Deciphering the role, or confirming the implication, of a candidate protein in a biological process often requires demonstrating that its expression is mandatory. *In vivo*, removal of a protein can be achieved by generating genetically modified mice in which the gene of interest has been deleted. Such knock-out animals may however be affected during development by adaptative compensatory mechanisms that may limit subsequent analysis of gene deletion (Sibille and Hen, 2001). Therefore, conditional knock-out approaches are often preferred in which excision of the target gene can be performed in a time and space dependent manner using cre recombinase activity (Sauer, 1998). One way to achieve this goal is to encode the enzyme on a recombinant virus for which spatio-temporal control of

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infection conditions and, hence, of cre-recombinase expression can be performed (Ahmed et al., 2004; Kaspar et al., 2002). Also, spreading of the viral infection is often not desired to ensure local targeting of cre-recombinase and spatially restricted knock out activity. In this context, a virus that raises few or no safety issues and infects a limited volume of target tissue is of interest. *Autographa californica* baculoviruses cannot replicate in mammalian cells (Hofmann et al., 1995) and are considered as safe viral vectors also for human gene delivery (Li et al., 2012). However, few attempts have been published regarding baculovirus-dependent gene delivery in neurons. So far, successful *in vivo* or *in vitro* expression was only described for three reporter genes: eGFP (Sarkis et al., 2000; Tani et al., 2003; Wang et al., 2007), luciferase (Li et al., 2004; Li et al., 2005; Wang and Wang, 2005; Wang et al., 2005), and β -galactosidase (Lehtolainen et al., 2002). Expression of the reporter proteins was detected in neurons and glial cells when driven by the human cytomegalovirus (CMV) or the CAG (comprising the CMV IE enhancer, chicken β -actin promoter and rabbit β -globin polyadenylation signal) ubiquitous promoters. Interestingly, the use of a hybrid promoter fusing the CMV enhancer to the human platelet-derived growth factor (PDGF) increased the tropism of *in vitro* and *in vivo* luciferase expression towards neurons but led to significant spreading outside the injection site through anterograde and retrograde axonal transports of the viral particles (Li et al., 2004; Wang and Wang, 2005). Recently, efforts have also been made to increase baculovirus infectivity towards mammalian cells by introducing surface glycoproteins from viruses naturally infecting these cells. The vesicular stomatitis virus glycoprotein (VSVG) significantly improved infection efficiency of several mammalian cell lines and enabled *in vitro* and *in vivo* infection of neurons as revealed by eGFP fluorescence (Tani et al., 2003). In this context, we evaluated a VSVG-modified recombinant baculovirus encoding the cre-recombinase under the control of the CAG promoter for its ability to efficiently excise a target sequence and for its potential to generate a conditional knock out in a brain area restricted to the infection site.

The baculoviral vectors pAcVSVG-CAG-luc and pAcVSVG-CAG containing the VSVG glycoprotein gene under the control of the polyhedrin promoter and, respectively, the luciferase gene or a *Bgl II* unique restriction site for cloning under the control of the CAG promoter were a generous gift from Dr Y. Matsuura (Osaka University, Japan) (Tani et al., 2001). A cloning cassette for expression under the CAG promoter was introduced by PCR in the pAcVSVG-CAG unique *Bgl II* site. The sequence corresponding to the cre recombinase fused to the eGFP at its C-terminal end was then introduced in the modified vector. Generation of recombinant baculoviruses AcVSVG-CAG-eGFP-Cre and AcVSVG-CAG-luc in *Spodoptera frugiperda* (Sf9), isolation and amplification of viral clones, as well as determination of viral titers were performed as described previously (Massotte et al., 1999).

COS (CV1 origin SV40) and Neuro2A cells were grown in DMEM medium supplemented with 10% fetal calf serum (FCS). Conditions of infection were optimized by infecting COS cells with AcVSVG-CAG-luc at a multiplicity of infection (MOI) ranging from 1 to 500. Luciferase activity was assayed 16h, 24h, 40h or 64h later (Luciferase bioluminescent assay, Promega) and presence of the protein determined by immunocytochemistry (Figure 1). Infection at a MOI of 50 for 24h was chosen for all subsequent experiments since it was characterized by high levels of enzyme and by low cell mortality. Cre-recombinase activity was then investigated. In order to visualize excision capability, we used a plasmid encoding the β -galactosidase preceded by a stop sequence flanked by two *loxP* sites under the control of the CMV promoter (pCMV-LNL- β Gal) (generous gift from D. Metzger, IGBMC). The plasmid was transfected using Jet-PEI according to the manufacturer's instructions in COS and Neuro2A cell lines (Figure 2). Transfected cells were infected with AcVSVG-CAG-eGFP-Cre. Both intranuclear eGFP fluorescence and X-gal staining (Sanes et al., 1986) indicated proper expression, nuclear targeting and enzymatic activity of the eGFP-cre-recombinase fusion construct (Figure 2 A-B). Similar results were obtained in HEK 293

cells (data not shown). AcVSVG-CAG-eGFP-Cre infection of Neuro2A neuroblastoma transfected with pCMV-LNL- β gal as described above also resulted in intranuclear eGFP fluorescence and positive X-gal staining (Figure 2 E-F). Data thus confirm efficient foreign gene delivery in mammalian cells by AcVSVG-CAG recombinant viruses as previously reported (Tani et al., 2003; Tani et al., 2001). Furthermore, these results establish that VSVG-modified recombinant baculoviruses are suitable to drive exogenous cre-recombinase activity in mammalian cells.

Next, we investigated AcVSVG-CAG efficiency for foreign gene delivery in primary neuronal cultures. Animal housing and experiments were conducted in accordance with the European Communities Council Directive of 26 May 2010 and approved by the local ethical committee (com'eth 2012-006). Primary neuronal cultures of the striatum were prepared from floxed ROSA 26 reporter mice pups (P0-P3) (generous gift from ICS) (Mao et al., 1999) as previously described (Pradhan et al., 2009; Scherrer et al., 2006). Cells were seeded at 10^5 cells/well on poly-L-lysine coated coverslips in 6-well plates and maintained in neurobasal A medium with 2% B27 supplement and 2 mM glutamine. On day 7, cultures were infected with AcVSVG-CAG at a MOI of 50 in serum-free Opti-MEM medium (Invitrogen). After incubation for 1 h, the supernatant was replaced with fresh culture medium. As previously reported, AcVSVG-CAG-luc infection led to luciferase detection in primary neurons (Li et al., 2004; Li et al., 2005; Wang and Wang, 2005; Wang et al., 2005) that we identified by the specific somatodendritic marker MAP2 (Figure 3). Upon infection with AcVSVG-CAG-eGFP-Cre, eGFP fluorescence was visible 24h post-infection in NeuN positive cells indicating efficient infection and protein synthesis in neurons in agreement with previously reported neuronal expression of this reporter gene (Tani et al., 2003; Wang et al., 2007). However, eGFP fluorescence was equally distributed between the nucleus and cytoplasm and no X-Gal staining could be detected (Figure 3). In order to verify the functionality of the eGFP-cre recombinase fusion construct in neurons, recombinant adeno-associated viruses (serotype 2) expressing the eGFP-Cre fusion protein under the control of the CAG promoter (AAV-CAG-eGFP-cre) were generated. Upon infection of primary neuronal cultures for 48h with 10^{10} genome copies, the observed intracellular distribution of the eGFP fluorescence was restricted to nuclei as expected (Figure 3). Interestingly, a similar proportion of neurons expressed eGFP following AcVSVG-CAG-eGFP-Cre ($72 \pm 5\%$, $n=3$) or AAV-CAG-eGFP-cre ($62 \pm 10\%$, $n=2$) infection indicating that AcVSVG-CAG recombinant viruses show high tropism towards neurons. Our data therefore confirm eGFP and luciferase gene delivery in primary neuronal cultures but suggest that AcVSVG-CAG-eGFP-Cre are unable to support cre-recombinase activity.

We then tested cre-recombinase expression in the mouse brain. AcVSVG-CAG-eGFP-Cre was injected in the striatum of 6 weeks-old floxed ROSA 26 mice (Paxinos Mouse atlas stereotaxic coordinates: AP +0.5 mm, LM +2 mm, DV +3 mm). Animals were infected with 0.5, 2 or 4 μ l (10^7 plaque forming units / μ l) AcVSVG-CAG-eGFP-Cre. This range of infective particles was chosen on the basis of previous *in vivo* successful expression of the reporter genes GFP and luciferase (Lehtolainen et al., 2002; Sarkis et al., 2000; Tani et al., 2003; Wang and Wang, 2005; Wang et al., 2005). Anesthetized animals were perfused intracardially with 4% paraformaldehyde 2, 7, 14, or 21 days post-infection ($n=2-3$ animals per condition). Both eGFP fluorescence and β -galactosidase activity were then investigated on brain sections. Based on eGFP fluorescence, the infection volume was estimated to be $5-6 \times 10^5 \mu\text{m}^3$. Similarly to what was observed in primary neuronal cultures, eGFP fluorescence was present mostly in the cytoplasm and no X-Gal staining was detected suggesting again limited nuclear localization of the enzyme (Figure 4 A-B). Similar results were obtained when infection assays were performed using a number of viral particles ranging from 10^5 up to 4×10^8 plaque forming units / μ l. Control injection of 4 μ l (2×10^8 genome copies / μ l) AAV-CAG-eGFP-cre was performed in the striatum of floxed ROSA

26 mice (n=3) using stereotaxic coordinates identical to AcVSVG-CAG-eGFP-Cre. Three weeks post-infection, both green fluorescence and X-Gal staining were detected demonstrating full functionality of the fusion construct (Figure 4 C-D).

Altogether, our data establish successful *in vitro* expression of the luciferase reporter gene in non-neuronal mammalian cells, neuroblastoma and mouse hippocampal primary neurons infected with AcVSVG-CAG recombinant baculoviruses. This confirms previous reports of luciferase expression using these recombinant baculoviruses in non-insect cells (Tani et al., 2003; Tani et al., 2001). Importantly, our results underline the intrinsic efficiency of VSVG-modified recombinant baculoviruses to target neuronal cells since their *in vitro* tropism towards neurons is comparable to what is observed following AAV infection.

In vivo, the volume of infection upon AcVSVG-CAG recombinant baculoviruses injection in the mouse brain remains limited. This is in line with a previous report in which baculoviruses expressing the β -galactosidase under the control of the CMV promoter resulted in few lac Z positive neurons when injected in the rat striatum (Lehtolainen et al., 2002). Both reports are consistent with infection by a virus that cannot replicate *in vivo* in mammalian cells (Hofmann et al., 1995) and emphasize their potential as safe viral vectors for human gene delivery (Li et al., 2012).

Finally, we report for the first time successful expression of a functional cre-recombinase and efficient excision of a target sequence following infection of mammalian and neuroblastoma cell lines with recombinant baculoviruses. VSVG-modified recombinant baculoviruses thus appear appropriate tools to generate conditional knock-out animals. More generally, our results demonstrate that VSVG-modified recombinant baculoviruses are suitable for efficient delivery of genes encoding proteins with nuclear activity in mammalian cells. However, despite expression of the protein, we were unable to detect enzymatic activity in primary neurons *in vitro* and in the mouse brain *in vivo* hampering brain directed applications. Additional developments are thus required to overcome this limitation and extend functional expression of nuclear proteins to neuronal cells.

Acknowledgments

A. Pradhan was supported by the INSERM-FRSQ (Fonds de la recherche en santé du Québec). We are grateful to the National Institute on Drug Abuse for supporting the Center for Opioid Receptors and Drugs of Abuse (#DA 005010). We also acknowledge funding from Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale and University Strasbourg.

We would like to thank Isabelle Cheynel and Jean-Luc Weickert (baculovirus), Pascale Koebel and Jean-Marie Garnier (AAV) for excellent technical assistance with viruses production.

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Highlights

- Cre-recombinase was expressed using a recombinant *A. californica* baculovirus
- Gene delivery was evaluated in mammalian cells, primary neurons and mouse brain
- Functional cre-recombinase was expressed in non-neuronal and neuroblastoma cells
- Non functional expression was detected in mouse primary neuronal cultures or brain

Figure 1. Luciferase expression in COS cells infected with AcVSVG-CAG-luc

Luciferase expression was detected 24h post infection at a MOI of 50 by immunocytochemistry using a rabbit polyclonal antibody (1:400, Sigma) revealed with a Fluorescein-conjugated secondary antibody (Jackson) (A). No expression was detected in cells infected with a wild type virus (C). Corresponding cells were identified by nuclear staining with DAPI (B, D). Scale bar 10 μ m.

Figure 2. eGFP-cre recombinase expression in COS or Neuro2A cell lines infected with AcVSVG-CAG-eGFP-Cre

Panel i: In the pCMV-LNL-βGal plasmid, a stop sequence flanked by two *loxP* sites is present between the CMV promoter and the β-galactosidase sequence. It prevents the expression of the enzyme. Recognition of the *loxP* sites by the cre-recombinase leads to the excision of the stop sequence, which enables β-galactosidase expression.

Panel ii: COS (A-D) and Neuro2A (E-H) cells were transfected with a plasmid encoding β-galactosidase sequence preceded by a floxed stop sequence. Cells were infected on the next day with AcVSVG-CAG-eGFP-Cre at a MOI of 50 for 24h. Expression of the fusion protein was detected by fluorescence (A, E). In a parallel experiment, cre recombinase activity was assessed by X-gal staining (B, F). No fluorescence or X-gal staining were detected in control non infected COS (C, D) or Neuro2A (G, H) cells. Scale bars 10 μm.

Figure 3. Luciferase and eGFP-cre recombinase expression in primary neurons infected with AcVSVG-CAG baculoviruses or AAV-CAG-eGFP-Cre adeno associated viruses

Panel i: Primary hippocampal cultures were infected with AcVSVG-CAG-luc baculoviruses at a MOI of 50 for 24h (A-D). Nuclei were stained with DAPI (A). Neurons were identified by MAP2 labeling (1:2000, Sigma) with a mouse monoclonal antibody revealed by an AlexaFluor 488 conjugated secondary antibody (1:500, Molecular Probes) (B). Luciferase expression was detected with a rabbit polyclonal antibody (1:400, Sigma) revealed with an AlexaFluor 594 conjugated secondary antibody (1:2000, Molecular Probes) (C). Colocalisation between MAP2 and luciferase stainings was observed (D). Primary hippocampal cultures were infected with AcVSVG-CAG-eGFP-Cre baculoviruses at a MOI of 50 for 24h (E-H) or with 10^{11} particles AAV-CAG-eGFP-cre for 48 h (I-L). Nuclei were stained with DAPI (E, I). eGFP-cre recombinase expression was detected with a rabbit polyclonal antibody raised against eGFP (1:1000, Invitrogen) revealed by an AlexaFluor 488 conjugated secondary antibody (1:2000, Molecular Probes) (F, J). eGFP was detected in the cytoplasm after baculovirus infection (arrowhead in F). Neurons were identified by NeuN labeling (1:100, Millipore) detected with a mouse monoclonal antibody revealed by an AlexaFluor 594 conjugated secondary antibody (1:500, Molecular Probes) (G, K). Colocalisation between neuN and eGFP stainings was observed (arrows in H, L). Scale bars 10 μ m.

Panel ii: Cre recombinase activity was assessed by X-gal staining in primary neurons infected with AcVSVG-CAG-eGFP-Cre baculoviruses at a MOI of 50 for 24h. The fluorescence associated to the eGFP-Cre fusion was visible in the cytoplasm (A) and no X-gal staining was detected in infected cells (B). Scale bars 10 μ m.

Figure 4. *In vivo* eGFP-cre recombinase expression upon infection with AcVSVG-CAG-eGFP-Cre or AAV-CAG-eGFP-cre

Rosa 26 mice were infected in the striatum with AcVSVG-CAG-eGFP-Cre baculoviruses (4×10^7 plaque forming units) (A, B) or AAV-CAG-eGFP-cre adeno-associated viruses (8×10^8 genome copies) (C, D). Following infection with the recombinant baculovirus, no X-gal staining was detected after infection (arrow in A) and eGFP fluorescence was observed mainly within the cytoplasm (B). In contrast, positive X-gal staining was observed after infection with the adeno-associated virus (arrow in C) and eGFP fluorescence was detected in the nucleus (arrows in D). Scale bars 50 μ m.