The GP64 protein of Autographa californica multiple nucleopolyhedrovirus rescues Helicoverpa armigera nucleopolyhedrovirus transduction in mammalian cells

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Autographa californica multiple nucleopolyhedrovirus (AcMNPV) belonging to the group I nucleopolyhedroviruses (NPVs) and expressing the envelope-fusion glycoprotein GP64 transduces a variety of mammalian cells to express foreign genes under the control of mammalian promoters. In contrast, the group II Helicoverpa armigera single NPV (HaSNPV) encoding a different envelope protein, the F protein, shows no detectable infectivity towards mammalian cells. This limitation was overcome by expressing AcMNPV GP64 in HaSNPV. Although the transduction ratios were lower overall, the range of mammalian cell types transduced by HaSNPV was consistent with those transduced by AcMNPV. These findings indicate that the F protein functions only in insect cells, whereas the GP64 protein works in both insect and mammalian cells.

INTRODUCTION

The family Baculoviridae comprises large, double-stranded DNA viruses that are pathogenic to invertebrates, infecting primarily insects of the order Lepidoptera (Blissard et al., 2000). Based on occlusion-body morphology, the family is subdivided into two genera, Nucleopolyhedrovirus (NPV) and Granulovirus (GV). NPVs can be further subdivided into groups I and II, according to phylogenetic analysis (Bulach et al., 1999; Chen et al., 1999; Herniou et al., 2001). During their replication cycle, baculoviruses produce two types of virion: occlusion-derived virus (ODV) and budded virus (BV). The latter mediates cell-to-cell virus spread in animals and in cell culture (Keddie & Volkman, 1985).

Group I NPVs, such as Autographa californica multiple nucleopolyhedrovirus (AcMNPV) and Orgyia pseudotsugata MNPV (OpMNPV), have GP64 as the major glycoprotein of the BV (Blissard & Rohrmann, 1989). GP64 is involved in BV binding to host cells, which is necessary and sufficient for the low pH-triggered membrane fusion during endocytosis (Blissard & Wenz, 1992; Hefferon et al., 1999). It is also essential for efficient virion budding from the cell surface (Oomens & Blissard, 1999). However, group II NPVs, as well as GVs, lack a homologue of the gp64 gene. Instead, envelope proteins unrelated to GP64 have been identified. These include Ld130 of Lymantria dispar MNPV (LdMNPV), Se8 of Spodoptera exigua MNPV (SeMNPV) and Ha133 of Helicoverpa armigera SNPV (HaSNPV). They are collectively known as fusion (F) proteins (Chen et al., 2001; Pearson et al., 2000). Like GP64, F proteins possess pH-triggered membrane-fusion activity (Pearson et al., 2000; Westenberg et al., 2002). Recent studies demonstrated that the F proteins of SeMNPV and LdMNPV could rescue gp64-null AcMNPV infectivity in an insect system (Lung et al., 2002). An F gene homologue, Ac23, which has lost its fusion function, has been also found in AcMNPV (Lung et al., 2003). The acquisition, exchange and evolution of such envelope proteins may dramatically affect the success and evolutionary divergence of viruses.

GP64 has a low but significant similarity to the envelope proteins of orthomyxoviruses from the genus Thogotovirus (Morse et al., 1992), a group of tick-transmitted viruses capable of replicating in both ticks and vertebrates. In contrast, F proteins share similarity with the envelope-fusion proteins of retroviruses (Pearson & Rohrmann, 2002). Analysis of complete baculovirus genome sequences has revealed that the baculovirus GP64 homologues are all closely related (>74% amino acid sequence identity), whereas the baculovirus F proteins are highly diversified (20–40% sequence identity) (Pearson & Rohrmann, 2002). Therefore, it has been suggested that GP64 has been acquired relatively recently to displace the baculovirus F proteins, such as Ac23, in group I NPVs (Pearson & Rohrmann, 2002).

AcMNPV can transduce mammalian cell lines, although it
replicates only in insect cells (Brusca et al., 1986; Carbonell & Miller, 1987). In the context of the baculovirus genome, a mammalian promoter drives the expression of a reporter in different mammalian cells (Hofmann et al., 1995; Liang et al., 2004). Enhanced transduction has been achieved in a variety of cell lines infected with recombinant AcMNPV expressing either vesicular stomatitis virus envelope G protein (VSV-G) or its envelope glycoprotein GP64 on the virion surface (Tani et al., 2001). GP64 is essential for AcMNPV to transduce mammalian cells. It is currently unknown whether group II NPVs can infect mammalian cell lines through the F protein.

In this study, we found that HaSNPV, a group II NPV with an F protein, failed to transduce mammalian cell lines. However, the gp64 gene could be used to rescue HaSNPV and enable it to transduce mammalian cell lines.

**METHODS**

**Cells and viruses.** *Helicoverpa zea* BCIRL-Hz-AM1 cells were cultured at 28 °C in Grace’s complete medium containing 10% fetal bovine serum (FBS; Gibco-BRL) (McIntosh et al., 1985). HaSNPV bacmid HaHZ8 was used as the original virus (Wang et al., 2003). Baculoviruses were amplified and purified from bacmid-transfected insect cells according to the BAC-to-BAC manual (Invitrogen) and the virus titre was determined by end-point dilution (O’Reilly et al., 1992). VSV India strain from the China Center for Type Culture Collection (CCTCC), Wuhan, China, was amplified in Vero E6 cells.

The mammalian cell types BHK (hamster kidney cell line), HepG2, Vero E6, HeLa, PK-15 and SMMC-7721 were obtained from CCTCC. BHK and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS. The other mammalian cells were maintained in RPMI 1640 medium with 10% FBS.

**Cloning of the envelope-protein genes.** Vero E6 cells were infected with VSV at an m.o.i. of 1. After cytopathic effects appeared in infected cells, the supernatant was collected. Viral RNA was extracted from the supernatant by using TRIzol reagent (Invitrogen). The primer VSVG3019 (5’-CCTGTCTTTTCTTATCCCTATG-3’) was used to synthesize the first-strand cDNA by using SuperScript II reverse transcriptase (Invitrogen) and PCR amplification was performed with the primers VSVG3068 (5’-CCTGTGACTATGAGGCTCAGCC-3’) and VSVG4621 (5’-TGAAAGTCTTGTGACGCG-3’) using the Advantage KlenTaq polymerase mix (Clontech). The PCR-amplified product was cloned into the pGEM-T Easy vector (Promega) to generate pT-vsvg. The AcMNPV gp64 open reading frame (ORF) was PCR-amplified with the primers Hind-gp64u (5’-GAAGCTTAAATGTTGTAAGGC-3’) and Hind-gp64d (5’-GAAGCTTTAATATTGTGCATTACGTTTCTA-3’), using AcMNPV bacmid as template. The PCR products were cloned into the pGEM-T Easy vector to produce pT-gp64.

**Construction of recombinant HaSNPV bacmids.** Several donor plasmids were generated for insertion of genes into the HaSNPV bacmid (HaHZ8) by Tn7-mediated transposition. All donor plasmids contained the green fluorescent protein (GFP) reporter gene under the control of the cytomegalovirus (CMV) IE1 promoter and some also contained the gp64 or VSV-G gene under the control of the 166 bp OpMNPV fusion promoter (ORF126) promoter (Op166; Blissard & Rohrmann, 1991). Fig. 1 shows the recombinant baculoviruses that were generated for these studies.

To generate HaSNPVs with dual envelope proteins, the gene (gp64 or VSV-G) encoding the heterogeneous viral envelope protein was cloned downstream of the promoter Op166 into modified pFastBac1 (Life Technologies) to generate pFBOp166-CMV-GFP. The plasmid pFastBac1 was first digested with BstI and BamHI to remove the polyhedrin gene promoter sequences and the CMV IE promoter/enhancer was inserted as a 0.8 kbp NrdI–BamHI fragment from pFBDNA3 (Invitrogen), and the enhanced GFP gene from pEGFP-I (Clontech) as a BamHI–Xhol fragment was then cloned into pFB-CMV under the control of the CMV promoter. This produced the recombinant plasmid pFB-CMV-GFP. pFBOp166-CMV-GFP was then constructed by cloning the cassette containing the promoter Op166 with the multiple cloning site and polyadenylation signal from pOP166AcV5 (provided by Just M. Vlak, University of Wageningen, The Netherlands) with blunt ends into the BclI site of pFB-CMV-GFP. The gp64 gene from pT-gp64 as an EcoRI fragment was then inserted downstream of the promoter Op166 in

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**Fig. 1.** Schematic diagram of the recombinant baculovirus constructs, showing the strategy for insertion of the envelope-protein gene construct into the polyhedrin locus of the HaSNPV bacmid. Inserts of a CMV IE1 promoter–GFP reporter without an envelope-protein gene (upper construct) or the Op166 promoter controlling the envelope-protein gene VSV-G (middle construct) or gp64 (lower construct) were performed. All cassettes depicted were inserted into the attB site (indicated by right and left insertion sites, Tn7R and Tn7L) in the polyhedrin locus by Tn-based transposition.
pFBop166-CMV-GFP to generate the donor plasmid pFB-gp64-CMV-GFP. The other fusion-protein gene, VSV-G, was also cloned into the same site to construct pFB-vsvg-CMV-GFP. The three donor plasmids (pFB-CMV-GFP, pFB-gp64-CMV-GFP and pFB-vsvg-CMV-GFP) mediated insertion of genes into the HaSNPV bacmid by Tn7-mediated transposition to generate the bacmids HaGP64-CMV-GFP, HaVSVG-CMV-GFP and Ha-CMV-GFP (Fig. 1), respectively.

The recombinant bacmids were transfected into Hz-AM1 cells by using Lipofectin (Invitrogen) to generate infectious virus. Virus was further amplified by propagation in Hz-AM1 cells grown in Grace’s supplemented insect medium containing 10% FBS. Virus titres were determined by plaque assay on Hz-AM1 cells using a standard method (O’Reilly et al., 1992).

**BV purification.** After amplification of each recombinant baculovirus, cell debris was removed by centrifugation at 1000 g for 10 min and virions were then concentrated from the cell-culture supernatant by centrifugation at 50 000 g for 1 h at 4 °C. The pellet containing virus was resuspended in PBS and used for transduction experiments. The recombinant BV was further purified by 25–60% linear sucrose-gradient centrifugation (90 000 g for 2 h). A white band formed at approximately 45% sucrose was collected and diluted 10 times in PBS. These virions were concentrated by centrifugation at 50 000 g for 1 h, resuspended in PBS and then used for SDS-PAGE and Western blotting.

**Baculovirus transduction of mammalian cells and GFP-expression assays.** Mammalian cells were seeded in six-well culture dishes at a concentration of 2 x 10⁵ cells per well. Culture medium was removed, replaced with virus inoculum with various m.o.i. values and incubated for 2 h at 37 °C. The inoculum was then replaced with 2 ml fresh medium with or without 10 mM sodium butyrate. Cultures were analysed for GFP expression by using fluorescence microscopy. For flow-cytometry (FACS) analysis, cultures were harvested with 0.5% trypsin, washed and resuspended in Dulbecco’s PBS with 1% FBS at 48 h after inoculation. Data collection was performed on a Beckman Coulter flow cytometer.

**Transfection assays.** DNA was prepared from 1-5 ml cultures of two to three independent colonies carrying the bacmid Ha-CMV-GFP or Ac-CMV-GFP according to the BAC-to-BAC manual (Invitrogen) and analysed in parallel. DNA (1-5 μg) from each preparation was used to transfect 2 x 10⁵ BHK cells in a six-well plate by using Lipofectin (Invitrogen) according to the manufacturer’s instructions. At 48 h post-transfection, transfected cells were observed by fluorescence microscopy to monitor expression of the CMV-GFP cassette.

**Immunostaining.** Hz-AM1 cells were seeded in 96-well plates at a concentration of 2 x 10⁴ cells per well. Culture medium was removed, replaced with 20 p.f.u. virus inoculum per well and incubated for 1 h at 28 °C. The inoculum was then replaced with 40 μl 0-6% (w/v) methycellulose in Grace’s medium plus 10% FBS in each well and incubated for 72 h at 28 °C. The methycellulose medium was washed with formyl-buffered acetone (30% PBS, 40% acetone, 9-25% formaldehyde). After cells had been blocked with normal goat serum, anti-GP64 mAb B12D5 (provided by L. E. Volkman, University of California, Berkeley, USA) was added to the wells. Cells were washed twice with PBS. Alkaline phosphatase-conjugated goat anti-mouse IgG was added to the wells to detect binding of mAb B12D5. NBT/BCIP was added to stain positive cells after unbound secondary antibody had been removed by washing with PBS. Purple cells were detected by microscopy.

**RESULTS**

**HaSNPV of the group II NPVs does not transduce mammalian cells**

To determine whether group II NPVs, which contain an F protein rather than GP64 as the envelope-fusion protein, could transduce mammalian cells, we constructed a recombinant HaSNPV (Ha-CMV-GFP) containing the GFP gene under the control of the CMV IE1 promoter (Fig. 1). We transduced a variety of mammalian cells (BHK, HepG2, HeLa, Vero and PK-15) with Ha-CMV-GFP BV at an m.o.i. of 50. In this experiment, Ac-CMV-GFP BV (Liang et al., 2004) was used as the positive control. Cultures were examined for GFP expression by fluorescence microscopy at 24, 48 and 72 h post-treatment. We could not detect a GFP signal in any of the Ha-CMV-GFP-transduced cell lines (results for BHK cells are shown in Fig. 2b). Moreover, a GFP signal was not detected when we added 10 mM butyrate, a chemical reagent that inhibits histone deacetylase (Condrey et al., 1999) (data not shown). In contrast, GFP expression was observed in all tested cells transduced by Ac-CMV-GFP (Fig. 2a) and the level of expression increased following the addition of 10 mM butyrate, as reported previously (Liang et al., 2004) (data not shown). Flow-cytometric analysis of all tested mammalian cell types treated with Ha-CMV-GFP also gave negative results (data not shown). These results indicated that Ha-CMV-GFP BV could not transduce or mediate GFP expression in the mammalian cells tested.

In contrast, BHK cells transfected with either Ha-CMV-GFP or Ac-CMV-GFP genome DNA did produce GFP-expression signals (Fig. 2e and f). Thus, the GFP cassette in the Ha-CMV-GFP genome was shown to be active in mammalian cells. The inability of HaSNPV to transduce mammalian cells thus seemed to be due to the failure of Ha-CMV-GFP to enter mammalian cells. It appeared that the envelope protein GP64 was essential for AcMNPV to enter mammalian cells (Tani et al., 2001), whereas the major envelope protein of HaSNPV, Ha133, could not mediate entry of HaSNPV into mammalian cells. We therefore designed a pseudotype HaSNPV expressing GP64 to help us address this question.

**AcMNPV GP64 is expressed and assembled into BVs of recombinant HaSNPV**

A gp64-pseudotyped lentiviral vector has been shown to be delivered efficiently into mammalian cells in vitro and in vivo (Schauber et al., 2004). Thus, we wanted to determine whether heterogeneous fusion proteins such as GP64 and VSV-G could rescue HaSNPV transduction in mammalian cells. We constructed two recombinant HaSNPV bacmids (HaGP64-CMV-GFP and HaVSVG-CMV-GFP) to detect whether heterogeneous fusion proteins could functionally rescue HaSNPV transduction of mammalian cells. Two donor plasmids containing the gp64 or VSV-G gene under the control of the Op166 promoter and the
The gfp gene under the control of the CMV IE1 promoter were constructed. The cassettes were transferred into the genome of the bacmid HaHZ8 by using the BAC-to-BAC system, resulting in the recombinants HaGP64-CMV-GFP and HaVSVG-CMV-GFP. After transfection of Hz-AM1 cells with the recombinant bacmid DNAs, BVs of HaGP64-CMV-GFP and HaVSVG-CMV-GFP were generated.

To determine whether the GP64 protein was expressed in infected cells and present in HaSNPV BVs, we performed Western blot analysis by using purified BVs of HaGP64-CMV-GFP. A band was detected by using the anti-GP64 mAb B12D5 (Fig. 3a). This band was also detected in the AcMNPV positive control, but not in the negative-control viruses HaSNPV and Ha-CMV-GFP (Fig. 3a). However, GP64 produced by HaGP64-CMV-GFP in Hz-AM1 cells was slightly larger than GP64 produced by AcMNPV in Sf21 cells (Fig. 3a). There may be alternative post-translational modification of GP64 in the two different virus/cell systems. In addition, at 48 h post-infection, immunostaining detected plaques in Hz-AM1 cells infected with HaGP64-CMV-GFP in which all cells were stained with the anti-GP64 mAb B12D5. Hz-AM1 cells were infected with Ha-CMV-GFP (b) or HaGP64-CMV-GFP (c). A plaque-forming unit expressing GP64 protein is indicated by an arrowhead in (c) in Hz-AM1 cells at 48 h after infection with HaGP64-CMV-GFP.

**AcMNPV GP64 rescues HaSNPV transduction into mammalian cells**

To examine whether the pseudotypes HaGP64-CMV-GFP and HaVSVG-CMV-GFP could rescue the entry of HaSNPV into mammalian cell types, we incubated BHK cells with HaGP64-CMV-GFP or HaVSVG-CMV-GFP-containing supernatant. Cells expressing GFP were observed in BHK cells 48 h post-treatment with HaGP64-CMV-GFP at an m.o.i. of 50 by using fluorescence microscopy (Fig. 4a). The level of GFP expression was analysed by flow cytometry. The percentage of GFP-positive cells in BHK cells 48 h after treatment with HaGP64-CMV-GFP at an m.o.i. of 50 by using fluorescence microscopy (Fig. 4a). The level of GFP expression was analysed by flow cytometry. The percentage of GFP-positive cells in BHK cells was 4.7 ± 0.43, which was nearly one-eighth of the number of cells transduced with Ac-CMV-GFP under the same conditions (Fig. 4c). Surprisingly, only a few cells were detected in BHK cells treated with HaVSVG-CMV-GFP under the same conditions (data not shown).
compound inhibits histone deacetylase, which induces hyperacetylation of chromatin and leads to the induction of expression of repressed genes (Condreay et al., 1999). At a concentration of 10 mM, sodium butyrate had a significant effect on GFP expression in BHK cells treated with HaGP64-CMV-GFP (Fig. 4b). Flow-cytometry analysis showed that the number of GFP-expressing BHK cells transduced with HaGP64-CMV-GFP with added butyrate was 11.3 ± 1.35%, nearly three times that in transduced BHK cells with no added butyrate (Fig. 4c). The level of GFP expression remained low in BHK cells transduced with HaVSVG-CMV-GFP in the presence of 10 mM butyrate, although the level was higher than without butyrate (data not shown).

It is obvious that the ability of the pseudotype HaSNPV, HaGP64-CMV-GFP, to transduce BHK cells is much lower than that of the recombinant AcMNPV (Fig. 4c). Both F protein and GP64 are present on these virions in different proportions, and this may contribute to the lower transduction efficiency.

Efficiency of HaGP64-CMV-GFP transduction into mammalian cells

As described above, HaGP64-CMV-GFP was found to be able to transduce BHK cells. In order to determine the transduction efficiency of different mammalian cell lines by HaGP64-CMV-GFP, we analysed the percentage of GFP-positive cells in various mammalian cell lines transduced with 10, 50 and 100 m.o.i. of virus, respectively. As the virus dose increased, the percentage of GFP-positive cells increased. The percentage of GFP-positive BHK cells was 2.01 ± 0.13, 4.68 ± 0.43 and 16.90 ± 1.1 for HaGP64-CMV-GFP at m.o.i.s of 10, 50 and 100, respectively (Fig. 5). Similar transduction efficiencies were found in the other mammalian cell lines tested (Fig. 5), and all were lower than that of AcMNPV transduction at the same m.o.i. (Condreay et al., 1999; Liang et al., 2004). In addition, the human hepatoblastoma cell line SMMC-7721 was tested (Tang et al., 2001; Yang et al., 2004). SMMC-7721 cells could not be transduced by HaGP64-CMV-GFP or Ac-CMV-GFP (data not shown), indicating that transduction of mammalian cells by AcMNPV is selective. We believe that this is probably due to the absence of the receptor on the surface of these cells.

DISCUSSION

Analysis of genomic sequences of baculoviruses has shown that all appear to have an F gene, whereas gp64 genes have been identified only in closely related viruses belonging to group I NPVs (Pearson & Rohrmann, 2002). GP64 is necessary for attachment and entry of the virus, as well as for assembly and budding of viral particles in the infectious cycle of AcMNPV (Blissard & Wenz, 1992; Hefferon et al., 1999), but can be replaced functionally by F proteins from group II NPVs, such as SeMNPV and LdMNPV, which do not have a gp64 gene (Lung et al., 2002). This indicates that the group II NPV F proteins are functionally analogous to
GP64. It has been reported that VSV-G protein can complement a GP64-null virus, indicating that VSV-G is functionally analogous to GP64 (Mangor et al., 2001). Recent experiments have indicated that AcMNPV can enter a broad range of mammalian cell lines (Boyce & Bucher, 1996; Condreay et al., 1999; Liang et al., 2004) and that it is taken up by mammalian cells in the same way as in insect cells, by receptor-mediated endocytosis, followed by pH-dependent fusion of the envelope with the endosome (Condreay et al., 1999). It was therefore particularly interesting to determine whether F proteins could also mediate baculovirus transduction of mammalian cells. We found that a recombinant HaSNPV (Ha-CMV-GFP) with the F protein as its fusion protein did not transduce a variety of mammalian cell lines. However, GFP was expressed in these cells following transfection with Ha-CMV-GFP genomic DNA, indicating that the baculoviral F protein could not mediate baculovirus entry into these mammalian cells. Thus, in mammalian cells, the F protein is not functionally analogous to GP64, which can mediate AcMNPV entry into mammalian cells. A recent study showed that GP64 mediated highly stable infectivity of a human respiratory syncytial virus lacking its homologous transmembrane glycoproteins (Oomens & Wertz, 2004), suggesting that GP64 can perform the function of mediating entry of baculoviruses or mammalian viruses into mammalian cells. In the present study, GP64 enabled HaSNPV BV possessing the F protein to enter mammalian cells. Moreover, the range of mammalian cell types transduced with HaGP64-CMV-GFP was consistent with that of AcMNPV. This indicated that GP64 was expressed and assembled on the surface of HaSNPV BVs and was the essential factor for baculovirus entry into mammalian cells. However, the pseudotype HaSNPV transduced all mammalian cells tested at lower ratios than AcMNPV. Moreover, another pseudotype HaSNPV containing the VSV-G protein, which possesses a strong membrane-fusion activity, had a weaker ability to transduce mammalian cells. This could be due to the GP64 or VSV-G protein being assembled into the pseudotype HaSNPV viral particles in low abundance or to the Ha133 protein blocking one or several motifs of these proteins that perform the function of fusion with mammalian cells.

Lung et al. (2003) recently reported that the viral pathogenic factor of infected insect hosts, Ac23, the F homologue in AcMNPV, was not required for virus replication or pathogenesis in cell culture or infected animals. This indicates that the F homologue in AcMNPV has lost the function of membrane fusion during evolution. Gp64-null AcMNPV pseudotyped with F proteins from LdMNPV and SeMNPV of group II NPVs generated infectious virions, although with lower virus titres than wild-type AcMNPV (Lung et al., 2002). These observations and our results suggest that GP64 might have been acquired from mammalian-related sources by an ancestor of the group I NPVs in the late stage of baculovirus evolution and replaced the envelope-fusion function of the baculovirus F homologue (Pearson & Rohrmann, 2002). In addition, GP64 is related closely (~30% identity at the amino acid level) to the envelope-fusion protein encoded by members of the genus Thogotoivirus (family Orthomyxoviridae), which are pathogenic for ticks and can also infect vertebrates (Pearson & Rohrmann, 2002). Thus, GP64 is similar to envelope-fusion proteins that function promiscuously in invertebrate and vertebrate cell types. In contrast, F proteins, which are more divergent and specific to baculovirus species, are apparently more specialized to promote BV entry into invertebrate cell types.

At present, the use of vectors for targeted gene delivery is a central issue within gene therapy. AcMNPV has been shown to be able to mediate gene transfer to a wide range of mammalian cells. Although the F protein does not mediate baculovirus transduction in mammalian cells, we were able to use GP64 to target HaSNPV to a number of mammalian cell types. This indicates that HaSNPV could be developed as a useful tool for mammalian cell-transduction experiments. The ability of fusion proteins to display ligands (Ojala et al., 2001, 2004) will allow further development of an HaSNPV gene-transduction system that is targeted to specific tissues. A ligand-displaying HaSNPV should not only infect insect cells to produce high titres of virus particles, but should also be highly efficient at transducing target mammalian cells.

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