Seamless replacement of *Autographa californica* multiple nucleopolyhedrovirus gp64 with each of five novel type II alphabaculovirus fusion sequences generates pseudotyped virus that fails to transduce mammalian cells

Marcel Westenberg, Helen M. Soedling, Nisha Hirani, Linda J. Nicholson, Derek A. Mann and Colin T. Dolphin

**INTRODUCTION**

The family *Baculoviridae* comprises large, rod-shaped, enveloped dsDNA viruses infective to insects belonging to the orders Lepidoptera, Hymenoptera and Diptera. Based on phylogenetic analysis of genome sequences, members of the family *Baculoviridae* are organized into four major genera: *Alpha*, *Beta*, *Gamma* and *Deltabaculovirus* (Jehle et al., 2006), with the former being subdivided into type I and type II alphabaculoviruses based, in part, on their use of the different major envelope fusion proteins, GP64 and fusion (F), respectively (Pearson & Rohrmann, 2002). From a historical perspective, baculovirus species have been used both as effective biological pesticides (reviewed by Szewczyk et al., 2006) and, in the form of the prototype *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), as a popular gene-delivery vector for heterologous protein expression in cultured insect cells (reviewed by van Oers, 2011). Proteins expressed in such an expression system can be generated at high yield, correctly folded and with many of the post-translational modifications, such as glycosylation, associated with mammalian cells (van Oers, 2011). Some time...
after these applications were established, it was discovered that AcMNPV was also able to transduce and deliver a functional transgene to a range of mammalian cells both in vitro and in vivo (reviewed by Hüser & Hofmann, 2003). Demonstrated originally in primary hepatocytes and liver-derived cell lines (Hofmann et al., 1995; Boyce & Bucher, 1996), the range of cells transduced by AcMNPV has since grown (see Chen et al., 2011) and now includes several additional primary cell types, including hepatic stellate cells (Gao et al., 2002), neural cells (Sarkis et al., 2000) and pancreatic islet cells (Ma et al., 2000). This unexpected competence for non-host-cell transduction has prompted researchers to design AcMNPV-based vectors for a variety of novel applications including assay development in drug discovery (Kost et al., 2010), RNA interference (Nicholson et al., 2005; Ong et al., 2005), cancer (Wang & Balasundaram, 2010), gene therapy (Ghosh et al., 2004) and engineering of stem cells in tissue regeneration (Lin et al., 2010). A possible regulatory step towards the eventual clinical application of AcMNPV-based vectors has been discussed recently (Lesch et al., 2011).

The broad range of non-host cells transduced by AcMNPV, together with the ability of the fusion protein GP64 to functionally replace fusion proteins from lentivirus (Schauber et al., 2004) and human respiratory syncytial virus (Oomens & Wertz, 2004), indicate that GP64 acts as a promiscuous fusion protein. Whilst such lack of selectivity is not relevant for AcMNPV-mediated gene delivery to cultured mammalian cells, it is less desirable if developing AcMNPV as a potential gene therapy vector because of unwanted bystander effects. Thus, it would be advantageous if AcMNPV-based vectors could be engineered with selective cell tropisms whilst retaining facile amplification to high titre in insect cell culture and the capacity to deliver a functional cargo to non-host cells. AcMNPV GP64 is necessary for viral attachment to (Heffron et al., 1999), membrane fusion during entry into (Blissard & Wenz, 1992; Kingsley et al., 1999) and subsequent budding from (Monsma et al., 1996; Oomens & Blissard, 1999) host insect cells. In addition, GP64 plays a critical role in mammalian cell transduction (Tani et al., 2001). A number of studies (Table 1) have demonstrated that the majority of type II alphabaculoviral F proteins, although apparently binding to a different insect cell receptor (Westenberg et al., 2007), can substitute functionally for GP64 by supporting AcMNPV production in culture, albeit with reduced titres. In contrast, F-pseudotyped Δgp64 AcMNPV vectors are severely compromised for mammalian cell transduction (Liang et al., 2005; Westenberg et al., 2007, 2010; Yu et al., 2009), confirming the key role that GP64 plays in non-host-cell transduction. However, the ability of F proteins to support Δgp64 AcMNPV in cultured host-cell entry and budding, plus the observation that they promote membrane fusion in an in vitro assay (Lung et al., 2002), indicate that the failure of F-pseudotyped viruses to transduce mammalian cells may result from a block at virus attachment or internalization rather than endosomal escape. Thus, as others have discussed (Yu et al., 2009), decorating the surface of F-pseudotyped Δgp64 AcMNPV vectors with, for example, cell-binding ligands or peptides represents a possible path towards generating AcMNPV-based gene therapy vectors with transductional targeting characteristics.

We recently developed (Westenberg et al., 2010) a modified counter-selection recombineering approach that enables us to undertake robust, seamless and unrestricted modification of the DNA sequence of the AcMNPV replicon bMON14272 (Luckow et al., 1993). This modified approach was necessary to overcome, during the counter-selection step, unwanted intra-molecular rearrangements that can occur between pairs of homologous regions present in the bacmid sequence (Westenberg et al., 2010). We are employing the technique as part of a strategy to generate AcMNPV-based vectors designed to deliver therapeutic genes and/or dsRNAs selectively to hepatic stellate cells in the context of liver fibrotic disease. Our initial aim is to identify, by replacing gp64 with a range of

### Table 1. F-pseudotyped Δgp64-AcMNPV

<table>
<thead>
<tr>
<th>F source*</th>
<th>Locus†</th>
<th>Promoter‡</th>
<th>Δgp64 rescue§</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SeMNPV</td>
<td>polh</td>
<td>gp64</td>
<td>Yes</td>
<td>Lung et al. (2002); Yu et al. (2009)</td>
</tr>
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<td>gp64</td>
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<td>Westenberg et al. (2010)</td>
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<td>gp64</td>
<td>Yes</td>
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<td>Yes</td>
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<tr>
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<td>Yes</td>
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</tr>
<tr>
<td>AgseGV</td>
<td>polh</td>
<td>gp64</td>
<td>Yes</td>
<td>Yin et al. (2008)</td>
</tr>
<tr>
<td>PlxyGV</td>
<td>polh</td>
<td>gp64</td>
<td>No</td>
<td>Lung et al. (2002)</td>
</tr>
</tbody>
</table>

*Source of F protein CDS. Type II alphabaculoviruses: SeMNPV, Spodoptera exigua MNPV; AdhoNPV, Adoxophyes honmai NPV; LdMNPV, Lymantria dispar MNPV; HearNPV, Helicoverpa armigera NPV. Betabaculoviruses: AgseGV, Agrotis segetum granulovirus; PlxyGV, Plutella xylostella GV.

†AcMNPV locus containing F CDS.

‡Promoter used to drive F CDS.

§Functionally substitutes for GP64.
sequences corresponding to the F-encoding ORFs from different type II alphabaculoviruses, an F-pseudotyped Δgp64 vector that can be cultured to high titre and lacks the promiscuous mammalian cell transduction characteristics of native AcMNPV. The envelope surface of this candidate F-pseudotyped vector will then be decorated with cell-binding ligands designed to provide selective cell targeting. Using this method, we previously reported (Westenberg et al., 2010) the seamless replacement of gp64 with F coding sequences (CDSs) from SeMNPV and AdhoNPV generating F-pseudotyped Δgp64 AcMNPV viruses that had lost the capacity to deliver a GFP transgene to mammalian cells. In the current study, we describe the construction of new Δgp64 AcMNPV vectors pseudotyped with five additional type II alphabaculovirus F proteins and report on their respective abilities in supporting virus production and the delivery of a reporter to mammalian cells.

RESULTS AND DISCUSSION

As a guide to F CDS selection, we undertook a molecular phylogenetic analysis of all type II alphabaculoviral F protein sequences available at the time and, by visual examination of the resulting phylogram (Fig. 1), selected the following five previously uncharacterized species that covered the major evolutionary nodes: ChchNPV (van Oers et al., 2005), SfMNPV (Harrison et al., 2008), SpltNPV strain G2 (Pang et al., 2001), MacoNPV-A (Li et al., 2002) and AgseNPV (Jakubowska et al., 2006). Using our modified recombinating protocol (Westenberg et al., 2010), the F CDSs were PCR amplified and, following addition of extended terminal sequences homologous to the gp64 5' and 3' flanking regions, via an intermediate subcloning step, each sequence was introduced into a Δgp64-bMON14272 bacmid (Fig. 2). This approach to generate F-pseudotyped AcMNPV resulted in the F CDS being placed in the equivalent genomic context as gp64 and differs from the more traditional method of utilizing Tn7-mediated transposition to insert sequences into the polyhedrin (polh) locus (Luckow et al., 1993). PCR and restriction enzyme analyses of all final bacmid sequences (Fig. S1, available in JGV Online) confirmed that our modified protocol, which employs terminal homology arms significantly longer (~600 bp) than the usually sufficient 50 bp, was both efficient – for each F pseudotype, at least three of five clones examined contained the desired F sequence (data not shown) – and, importantly, generated products free of the intra-molecular deletions that can otherwise commonly occur in the repetitive bMON14272 target during counter-selection recombinating (Fig. S1) (Westenberg et al., 2010). The resulting Δgp64>F bacmids and the control Δgp64 bacmid were each subsequently fitted, by standard Tn7 transposition, with a GFP reporter sequence driven by a hybrid cytomegalovirus (CMV)–p10 promoter (Westenberg et al., 2010), enabling facile visual monitoring of both Sf9 insect cell infection and mammalian cell transduction.

A transfection/infection assay (Fig. 3a, b) confirmed the ability of all the F pseudotypes to mediate viable infections from transfected Sf9 cells, confirming that all the F proteins investigated in this present study could functionally replace GP64. Thus, to date, it appears that the ability to functionally replace GP64 may be a universal feature common to all F proteins from type II alphabaculoviruses (Table 1). In contrast, of the two F proteins investigated
containing a different and discrete recombination method (In-Fusion; Clontech), generating plasmids introduced into SnaI and 39 rpsl-tetA(C) replaced the counter-selection (~50 bp) AcMNPV gp64 lAcMNPV bacmid bMW009, via FPCR amplicons, containing individual SnaI gp64 with negative selection, to generate a series of bacmids in which virus production in culture (Table S1) with titres ranging from 1.9 x 10^6 to 1.5 x 10^9 p.f.u. ml^-1 for the ChchNPV (vMW023) and MacoNPV (vMW021) F pseudotypes, respectively. In comparison, the AcMNPV GP64 rescue virus (vMW033) was amplified to ~10^9 p.f.u. ml^-1. Thus, although the titre was tenfold less than the GP64 rescue virus, the MacoNPV F-pseudotyped virus amplified to a useable concentration and this pseudotype has become our working vector for further development. Subsequently, in order to generate sufficiently concentrated virus stocks to enable mammalian cell transduction at virus:cell ratios close to 100, additional amplifications, with empirical combinations of starting cell densities and m.o.i. (data not shown), were carried out for those pseudotypes, such as ChchNPV F, that produced low titres in the first, controlled amplification. These provided working virus stocks for all F pseudotypes of ≥2.0 x 10^7 p.f.u. ml^-1 apart from ChchNPV and SpltNPV, which remained difficult to amplify to titres above those first recorded (Table S1).

Having generated working viral stocks, we then investigated whether any of the F pseudotypes were able to transduce either of the human cell lines Saos-2 and HeLa, which are readily transduced by native AcMNPV. Following incubation of Saos-2 cells with SeMNPV, AdhoNPV, MacoNPV or AgseNPV F-pseudotyped virus, fluorescence microscopy identified only very occasional, discrete GFP-expressing cells in each case (Fig. 3d). Apart from these isolated cells, no other GFP-expressing Saos-2 cells were observed. In HeLa cells, the situation was even more marked, as extensive searching failed to observe even occasional GFP-expressing cells after incubation with any of the F-pseudotyped viruses (Fig. 3f). As expected, the GP64 rescue vector transduced both Saos-2 and HeLa cells with efficiencies that were routinely >90 % (Fig. 3 and data not shown). Therefore, although all the F proteins tested here were able to substitute functionally for GP64 and support virus generation, the loss of the native envelope fusion protein essentially abolished the capacity for non-host-cell transduction, confirming the key role that GP64 plays in this process.

A number of studies have reported on investigations into the attachment and entry mechanisms involved during mammalian cell transduction with native GP64-equipped AcMNPV. Whilst early studies indicated roles for simple electrostatic interactions (Duisit et al., 1999) and phospholipids (Tani et al., 2001), later reports proposed the involvement of clathrin-mediated endocytosis (Long et al., 2006b) and macropinocytosis (Matilainen et al., 2005). More recently (Laakkonen et al., 2009), it has been suggested that neither of these latter mechanisms is significantly involved and that, instead, entry of AcMNPV into mammalian cells is mediated via a phagocytosis-like uptake process involving large plasma membranous invaginations associated with raft structures. Interestingly, experimental evidence has also been provided (Dong et al., 2010), demonstrating that incubation at low pH promotes AcMNPV uptake into both host Saos-2 and non-host mammalian cells via an endosome-independent pathway.

Fig. 2. Engineering of type II alphabaculovirus F protein-pseudotyped Δgp64 AcMNPV bacmids. pMW009 contains ~600 bp of AcMNPV gp64 5’ and 3’ flanking sequences fused together with a unique, centrally located SmaI restriction site. PCR amplicons, containing individual F CDSs flanking by short (~50 bp) AcMNPV gp64 5’ and 3’ flanking sequences, were introduced into SmaI-linearized pMW009, via an in vitro recombination method (In-Fusion; Clontech), generating plasmids containing a different and discrete F CDSS flanked by exact gp64 5’ and 3’ flanking sequences. These cassettes, linearized with SmaI, replaced the counter-selection rpsl-tetA(C) cassette in the Δgp64 AcMNPV bacmid bMW009, via λ Red-mediated recombineering with negative selection, to generate a series of bacmids in which gp64 is seamlessly replaced by different F CDSs (Westenberg et al., 2010).

from betabaculoviruses, only the fusion protein from AgseGV was demonstrated to be functionally analogous to GP64 (Yin et al., 2008) (Table 1).

We used a semi-quantitative assay as a means of directly comparing the ability of each of the five F-pseudotyped viruses generated in this study, plus two generated previously and the Δgp64>gp64 rescue vector (Westenberg et al., 2010), to functionally replace GP64. All viruses were amplified in Sf9 cells under equivalent conditions of culture and m.o.i., and titres were determined at 5 days post-infection (p.i.). All F-pseudotyped bacmids and the gp64 rescue bacmid were able to support virus production in culture (Table S1) with titres ranging...
involving direct membrane fusion at the cell surface. In contrast to these studies involving AcMNPV, there is little, if any, data available that sheds light on the block in mammalian cell transduction observed with F-pseudotyped AcMNPV that we, and others, have reported. As a first step towards this, we undertook a preliminary capsid immunolabelling experiment to investigate the fate of MacoNPV F-pseudotyped AcMNPV following incubation with Saos-2 cells (Fig. 4). Soon after incubation, immunostaining at the cellular periphery was observed with both the MacoNPV F-pseudotyped virus and a gp64 rescue virus, indicating that the former bound to the cell membrane. The lack of subsequent GFP expression in the cells bound by this pseudotype suggested a block at some subsequent point in the route normally followed by GP64-equipped virus. Further experiments are required to expand and extend this observation including the use of additional pseudotypes and cell lines and investigations to examine the effect of lowering the pH during cell–virus incubation.

The modified counter-selection recombineering method described by us previously (Westenberg et al., 2010) that enables precise and seamless modification of the AcMNPV genome has been further validated by the work detailed in the present report. The optimized methodology will enable others to undertake targeted modifications to the AcMNPV genome ranging from the types of seamless CDS swaps reported here down to subtle, single base-pair deletions,

Fig. 3. Sf9 transfection/infection and mammalian cell transduction with F-pseudotyped ∆gp64 AcMNPV viruses. Sf9 cells (a, b) were transfected (a) with ∆gp64 AcMNPV bacmid DNAs in which the gp64 locus was empty (bMW024, column 1) or had been seamlessly replaced with either a rescuing native gp64 CDS (bMW033, ∆gp64>gp64 AcMNPV, column 2), or the major envelope fusion CDS F from SeMNPV (bMW011, column 3), AdhoNPV (bMW036, column 4), ChchNPV (bMW023, column 5), MacoNPV (bMW021, column 6), AgseNPV, bMW035, column 7), SplitNPV (bMW034, column 8) or SIMNPV (bMW022, column 9) and, at 5 days post-transfection, inspected by fluorescence microscopy for GFP expression. An aliquot of the medium from each culture was used to infect a new batch of Sf9 cells (b) and, 3 days p.i., inspected for GFP expression. Cultures of either Saos-2 (c, d) or HeLa (e, f) cells were cultured (1 h, 28 °C) with clarified, filter-sterilized medium (500 µl) containing either the GP64-rescue (column 2) or F-pseudotyped (columns 3–9) virus and cells were visualized using bright-field (c, e) or fluorescence microscopy (d, f) at 2 days after incubation. For both cell types cultured with an F-pseudotyped virus, if fluorescence microscopy revealed a rare GFP-expressing cell(s), this field of view is provided. A black image indicates no GFP-expressing cells were detected. Magnification ×20. For bacmid construction, see this study, except for bMW033, bMW011 and bMW036 (Westenberg et al., 2010).
insertions or changes. We are employing this approach to furnish the surface of MacoNPV F-pseudotyped AcMNPV with cell-binding ligands designed to provide desired cell-targeting properties. If successful, such a strategy would represent an advance towards creating potentially clinically useful AcMNPV-based gene therapy vectors.

METHODS

Phylogenetic analysis. Type II alphabaculovirus F and AcMNPV gp64 amino acid sequences were aligned using CLUSTAL W (Thompson et al., 1994), within MacVector sequence analysis software (MacVector Inc.), and the resulting multiple sequence alignment file was optimized by eye. Using this file as input, phylogenetic analysis (again within MacVector) was undertaken, via alignment file was optimized by eye. Using this file as input, phylogenetic analysis (again within MacVector) was undertaken, via neighbour joining with systematic tie-breaking, and the resulting phylogenetic analysis was performed with CellSens (Olympus) and ImageJ (NIH) packages.

Plasmid construction and bacmid recombineering. Sequences encoding the F proteins of ChchNPV, SfMNPV, SpltNPV, MacoNPV-A and AgseNPV were PCR amplified (Phusion; NEB) using 5 ng of the corresponding viral genomic DNA as template and the respective PAGE-purified oligonucleotide (ODN) pairs 6021a/6022a, 6017a/6018a, 6108/6109, 6104/6105 and 6013a/6014a (Table S2). Each resulting amplicon contained the individual F CDS flanked by 62 bp of 5’ and 46 bp of 3’ sequence equivalent to regions immediately flanking the gp64 CDS of AcMNPV. The five amplicons were each cloned directly into SmalI-linearized pMW009, the construction and utility of which has been described elsewhere (Westenberg et al., 2010), via an in vitro recombination system (In-Fusion; Clontech) according to the manufacturer’s protocol, generating plasmids pMW025 (ChchNPV F), pMW026 (SfMNPV F), pMW031 (SpltNPV F), pMW030 (MacoNPV F) and pMW032 (AgseNPV F) (Table S3). Following DNA sequencing to confirm construct fidelity, the cloned sequences were released from their respective constructs by restriction with Smal, generating F CDS-containing, recombineering-ready cassettes, which were gel purified and quantified by visualization against a DNA molecular mass ladder (2-log ladder; NEB). In addition, a control, Δgp64 Smal restriction fragment, containing only 5’ and 3’ gp64 flanking sequences, was released from pMW009.

Each cassette, now equipped with ~600 bp flanking homology arms, was used to replace the rpsL-tet(A) counter-selection cassette (RT cassette) (Stavropoulos & Strathdee, 2001) in bacmid bMW009, in which the AcMNPV gp64 CDS has been directly replaced by the RT cassette, via counter-selection recombineering, as described previously (Westenberg et al., 2010). Briefly, aliquots (~0.1 ml) of bMW009-containing Escherichia coli MW001 cells (Westenberg et al., 2010), induced for phage λ Red activities and made electrocompetent, as described previously (Dolphin & Hope 2006), were electroporated with one of the five F CDS-containing cassettes or the Δgp64, non-F CDS-containing control cassette (Smal fragment of pMW009) (500 ng each), recovered (in SOC medium without MgSO4 at 32 °C with shaking at 220 r.p.m. for 2.5 h) and serially diluted (in 96 plates), and aliquots (50 μl) were spread on selective (containing 50 μg kanamycin (Kan) ml⁻¹, 500 μg streptomycin (Sm) ml⁻¹) or non-selective (containing 50 μg Kan ml⁻¹) NSLB agar (Stavropoulos & Strathdee, 2001) plates and incubated at 32 °C for 48 h. For each cassette, five discrete SmR colonies were streaked onto fresh Kan/Sm-selective plates, incubated at 32 °C for 48 h and subjected to colony PCR to identify clones in which the RT cassette had been replaced with an F CDS. Subsequent restriction digestion analyses of the resulting PCR products were performed to confirm the integrity of the F CDS replacement sequence (Fig. S1). Single bacmid clones, containing the correctly inserted ChchNPV, SfMNPV, SpltNPV, MacoNPV or AgseNPV F CDS or control sequence were named, respectively, bMW019, bMW018, bMW026, bMW017, bMW027 and bMW020 (Table S3). Bacmid DNAs were isolated and subjected to restriction mapping together with bMON14272, bMW009 and the previously generated bacmids bMW010, bMW028 and bMW025 (Westenberg et al., 2010) in which the native gp64 CDS was replaced by, respectively, SeMNPV F, AdhoNPV F and rescue AcMNPV gp64 CDSs (Fig. S1). Finally, each of the nine homologous regions (hr1, hr1a, hr2, hr2a, hr3, hr4a, hr4b, hr5a and hr5b) was PCR amplified with flanking ODNs, as described previously (Westenberg et al., 2010), and compared electrophoretically with equivalent PCR products amplified from bMON14272 (data not shown).

Following transformation with the Tn7-transposase helper plasmid pMON7124 (Luckow et al., 1993), bacmids bMW017, bMW018, bMW019, bMW026, bMW027 and bMW020 in E. coli MW001 received, via Tn7-mediated transposition (Bac-to-Bac; Invitrogen), the CMV_prom10+GFP dual insect/mammalian GFP reporter sequence from pMW005 (Westenberg et al., 2010), generating the respective bacmids bMW021, bMW022, bMW023, bMW034, bMW035 and bMW024 (Table S3).

Virus generation and mammalian cell transduction. Sf9 insect cells were passaged in shaking cultures (30 ml, 90 r.p.m., 28 °C) in serum-free medium (S9000 SFM; Invitrogen) supplemented with 2% (v/v) FBS. For virus generation, Sf9 cells (1.0 × 10⁶) grown in monolayers were transfected (Cellfectin; Invitrogen) with bacmid DNA (~1 μg), cultured (5 days, 28 °C) and inspected for GFP expression by fluorescence microscopy. An aliquot (500 μl) of the clarified (10 min, 10000 g), filter-sterilized (0.45 μm) medium was used to infect a new batch of Sf9 cells (1.0 × 10⁷). At 3 days p.i., cells were inspected for GFP expression. Subsequently, cells were passaged every 4–5 days until all cells were infected, at which point titres were
determined by an end-point dilution assay (O'Reilly et al., 1992). Quantitative determination of relative titre generation was performed by infecting S9 cells (1.0 × 10^6 cells ml^{-1}) in a shaking culture (30 ml, 90 r.p.m.) at an m.o.i. of 0.05. Cultures were harvested at 5 days p.i. and centrifuged (10 min, 1000 g) and the supernatants were filter sterilized. Titres were determined and the supernatants were stored at 4 °C. Individual bacmid constructs generated the corresponding ‘v’-prefixed virus (Table S3).

Soas-2 and HeLa cells were maintained (37 °C, 5 % CO_{2}) in Dulbecco’s modified Eagle medium (DMEM), supplemented with 10 % FBS. Cells were seeded (1.0 × 10^3) in 24-well plates and, after incubation (24 h), the medium was replaced with 500 μl clarified, filter-sterilized virus-containing S900II SFM resulting in virus : cell ratios of 50 (vMW022 and vMW023) or 100 (vMW011, VMW021, vMW033, vMW034, vMW035 and vMW036). After 1 h incubation at 28 °C with gentle shaking, the medium was aspirated, the cells were washed and fresh DMEM or Eagle’s minimal essential medium was added as appropriate. GFP expression was visualized in transduced cells by fluorescence microscopy (magnification × 20) at 48 h post-transduction.

**Immunohistochemistry.** Soas-2 cells (1.0 × 10^5), seeded on poly-L-lysine-coated coverslips placed in the wells of a 24-well plate, were cultured for 14 h, as described above, cooled to 4 °C and incubated with vMW033 (Ag64d>vgp64 rescue vector) or vMW021 (MacoNPV F-pseudotyped AcMNPV) for 1.5 h at 4 °C with gentle shaking at a virus : cell ratio of 500, after which they were washed twice with ice-cold PBS and fixed immediately (4 % formaldehyde, room temperature, 20 min). After fixation, the cells were washed three times with PBS, permeabilized with 0.2 % Triton X-100 in PBS for 10 min, washed again three times with PBS and blocked with 10 % goat serum in PBS for 2 h. The cells were then incubated for 14 h at 4 °C with an anti-VP39 mAb (Whitt & Manning, 1988) diluted 1:200 in 1 % goat serum in PBS. Cells, incubated with antibody only, were used. Cells were finally washed twice with PBS, washed again three times with PBS, permeabilized with 0.2 % Triton X-100 in PBS for 10 min, washed three times with PBS and incubated with 10 % goat serum in PBS for 2 h. The cells then were incubated for 14 h at 4 °C with an anti-VP39 mAb (Whitt & Manning, 1988) diluted 1:200 in 1 % goat serum in PBS, washed three times with PBS and incubated for 1 h at 37 °C with Cy3-conjugated goat anti-mouse antibody (Catlag Laboratories) diluted 1:200 in 1 % goat serum in PBS. As controls for specific antibody binding, mock-transduced cells, incubated with both antibodies, and transduced cells, incubated with the secondary antibody only, were used. Cells were finally washed twice with PBS, incubated for 30 min with Hoechst 33342 (10 μM in PBS; Sigma) and washed again twice with PBS. Coverslips were mounted in Fluorescence Mounting Medium (Dako) and the cells imaged (magnification × 100).

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