FUNCTIONAL ANALYSIS OF TWO BACULOVIRUS ENVELOPE PROTEINS

IAN-LING YU

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Department of Biological Sciences
University of Lethbridge
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Abstract

Budded virions of *Ac*MNPV can enter a variety of non-host cells, a characteristic likely due to the presence of GP64, an envelope protein found on a small subset of baculoviruses. Results show that *Ac*MNPV's tropism for vertebrate cells can be restricted - a prerequisite for using *Ac*MNPV for targeted *in vivo* gene delivery - by replacing the *gp64* gene with *SeF* from *Se*MNPV. Unlike the relatively well characterized GP64 protein, the significance and function of the F homolog (Ac23, a pathogenicity factor), is poorly understood. How Ac23 might contribute to the faster speed of kill was examined by comparing occlusion bodies and occlusion-derived virions (ODV) of Ac23null mutant viruses with control viruses at the ultrastructural level. The results show that Ac23null mutant produces a significantly higher percentage of ODVs with single or lower number of nucleocapsids than controls, suggesting Ac23 may play a role in multicapsid envelopment of ODVs.

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List of Abbreviations

293	Human embryo kidney cell line
A427	Human lung carcinoma cell line
Ac23	*Ac*MNPV F homolog
*Ac*MNPV	*Autographa californica* multicapsid nucleopolyhedrovirus
AFP	Alpha-fetoprotein
Amp	Ampicilline resistance gene
β-gal	*β*-galactosidase
BEV	Baculovirus expression vector
BHK	Syrian hamster kidney cell line
BCIP	5-bromo,4-chloro,3-indolylphosphate
*Bm*NPV	*Bombyx mori* nuclear polyhedrosis virus
BT	Bovine turbinate cell line
BV	Budded virus
CAG	Cytomegalovirus enhancer/chicken β-actin
CAT	Chloramphenicol acetyltransferase
CFBS	Characterized fetal bovine serum
CHSE-214	Fish salmon embryo cell line
CHO	Chinese hamster ovary cell line
CMV	Cytomegalovirus
COS-7	SV 40-Transformed african green monkey kidney fibroblast cell line
CPK	Piglet kidney cell line
CV-1	African green monkey kidney cell line

CTD	Cytoplasmic tail domain
DAF	Human decay-Accelerating factor
DAPI	4',6'-diamidino-2-phenylindole dihydrochloride
DFBS	Defined fetal bovine serum
DIC	Differential interference contrast
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
D-PBS	Dulbecco's phosphate-buffered saline
dpt	Day post transfection
dpi	Days post infection
eGFP	Enhance green fluorescent protein
EPC	Fish carp epidermal epithelioma cell line
*Eusi*NPV	*Euproctis similes* nucleopolyhedrovirus
F	Fusion protein
Flt-1	*fms*-like tyrosine kinase-1 promoter
FS-L3	Porcine kidney epithelial cell line
Gm	Gentamycin resistance gene
GP64	*Ac*MNPV envelope fusion protein
GUS	β-glucuronidase
GV	*Granulovirus*
*Hear*NPV	*Helicoverpa armigera* nucleopolyhedrovirus
HeLa	Human cervix carcinoma cell line

HepG2	Human hepatoma cell line
hFIX	Human factor IX
HMDS	Hexamethyldisilazane
hpi	hour post infection
hpt	hour post transduction
hrGFP	Humanized renilla green fluorescent protein
Huh7	Human hepatoma cell line
K562	Human erythromyeloblastoid leukemia cell line
L929	Strain L cloned fibroblast-like mouse cell line
LacZ	Bacterial beta -galactosidase gene
LCL-cm	Human lymphoblastoid B cell melanoma cell line
*Ld*F	*Lymantria dispar* multiple nucleopolyhedrovirus fusion protein
*Ld*MNPV	*Lymantria dispar* multiple nucleopolyhedrovirus
LTR	Long terminal repeat
Luc	Luciferase
MDBK	Madin-Darby bovine kidney cell line
MEM	Minimum essential medium
MKC	Primary mouse kidney cells
MNPV	Multiple nucleocapsid polyhedrovirus
moi	Multiplicity of infection
MSC	Mesenchymal stem cell line
*Neab*NPV	*Neodiprion abietis* nulceopolyhedrovirus
*Nele*NPV	*Neodiprion Lecontei* Nucleopolyhedrovirus

*Nese*NPV	*Neodiprion sertifer* nucleopolyhedrovirus
NIH 3T3	Mouse NIH Swiss embryonic fibroblast cell line
NBT	Nitroblue tetrazolium
NC	Nucleocapsid
NPV	*Nucleopolyhedrovirus*
OB	Occlusion body
ODV	Occlusion derived virus
Op21	*Op*MNPV F homolog
*Op*MNPV	*Orgyia pseudotsugata* multicapsid nucleopolyhedrovirus
ORF	Open reading frames
PBS	Phosphate buffered saline
r	Pearson correlation coefficient
R^2	The goodness of fit
Raji	Human African maxilla Burkitt's lymphoma cell line
Raw264.7	Murine macrophage cell line
RSV	Rous sarcoma virus
sCR1	Soluble complement receptor type 1
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning Electron Microscopy
*Se*MNPV	*Spotoptera exigua* multiple nucleopolyhedrovirus
*Se*F	*Spotoptera exigua* multiple nucleopolyhedrovirus fusion protein
SNPV	Single nucleocapsid polyhedrovirus
TEM	Transmission Electron Microscopy

Tn*7*	Tn*7* transposition site
TSA	Trichostatin A
U937	Human leukemic monocyte lymphoma cell line
VSV-G	Vesicular stomatitis virus G protein
WSSV	White spot syndrome virus
wt	Wildtype
X-gluc	X-glucuronide

Chapter 1 – Introduction of baculovirus

1.1 Baculovirus overview.

1.1.1 Structure and classification.

The Baculoviridae is a large family of enveloped viruses that are pathogenic to arthropods, primarily insects of the orders Lepidoptera, Hymenoptera, and Diptera (Federici, 1999). Baculoviruses have a large, double-stranded, covalently closed, circular DNA genome (Summers and Anderson, 1972). The size of the genome varies between species and can range from 80-180 kilobase (kb) in length (Burgess, 1977). The *baculo* portion of baculovirus refers to the rod-shaped nucleoprotein complex called nucleocapsids found in the viral particles (virions). Baculovirus nucleocapsids are typically 40-50 nm in diameter and 200-400 nm in length (Harrap, 1972), but can be extended to accommodate the larger genomes found in recombinant viruses carrying large inserts (Fraser, 1986). Baculoviruses are currently divided into two genera: *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV). NPV virions could either contain a single nucleocapsid (single nucleopolyhedrovirus, SNPV) or multiple nucleocapsids (multiple nucleopolyhedrovirus, MNPV) depending on the viral species (Theilmann *et al.,* 2005), while GV virions contain a single copy of the viral genome in a single nucleocapsid. Recently, a new classificantion was proposed based on phylogenetic evidence and further biological and morphological characteristics (Jehle *et al*., 2006). The family *Baculoviridae* was subdivided into four genera: Alphabaculovirus, Betabaculovirus, Gammabaculovirus, and Deltabaculovirus.

1.1.2 Infection cycle of *Ac*MNPV.

Among the baculoviruses, the infection cycle of the type baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus (*Ac*MNPV) is best characterized. In a single *Ac*MNPV infection cycle, virions with two distinct phenotypes and functions are produced. Budded virus (BV) are formed early in the infection cycle when nucleocapsids assembled in the nucleus are transported to the plasma membrane, and acquire an envelope by budding from the plasma membrane of the infected cell. The envelopes of BV contain envelope fusion proteins that mediate virus entry into cells by adsorptive endocytosis (Volkman and Goldsmith, 1985; Wang *et al.,* 1997). BVs are capable of infecting most cell types within a susceptible host and are thus responsible for spreading the infection systematically within a host. In the late phase of infection, nucleocapsids are no longer transported to the plasma membrane, but are enveloped within the nucleus to form occlusion-derived viruses (ODV). ODVs are eventually occluded in a matrix of polyhedrin protein to form an occlusion bodies (OB). OBs are released from the infected cells when the cell lyses, and the polyhedrin matrix is believed to protect ODVs from detrimental environmental and chemical insults, and help preserve ODV infectivity in the field. Upon ingestion of OBs by a susceptible host, OBs dissolve in the host's midgut, releasing the ODVs which are highly infectious to midgut epithelial cells. Thus, ODVs are the virion phenotype that is responsible for infection of larval arthropods in the field and for host to host transmission of viral infection.

1.1.3 *Ac*MNPV-based protein expression system.

*Ac*MNPV has become an important tool in both basic and applied research. The most

common application of *Ac*MNPV to date is its use as a tool for expression of recombinant proteins. Two decades ago, *Ac*MNPV was first used as an expression vector to express human beta interferon in insect cells (Smith *et al.,* 1983). Since then, numerous recombinant proteins have been produced using the baculovirus-insect cell expression system. Baculovirus expression vector (BEV) system is considered one of the best tools currently available for the expression of recombinant genes in a eukaryotic host. Unlike prokaryote-based expression systems, the eukaryote-based BEV system can produce proteins that are post-translationally modified by proteolytic cleavages, glycosylation, and phosphorylation (Jarvis, 1997). The foreign gene to be expressed is usually placed under the transcriptional control of a strong viral promoter such as the p10 or polyhedrin promoter, which enables the expression of the gene after viral infection of the host cells. In addition to the hundreds of different recombinant proteins which have been produced using the BEV system for research use, this system is now being used for the production of more than two dozen pharmaceutical proteins and vaccines that are either commercially available or undergoing clinical trials. For example, a BEV is used in the manufacture of human vaccines such as GlaxoSmithKline's cervical cancer vaccine Cervarix®, and Protein Sciences Corp.'s influenza vaccine FluBlok™, as well as animal vaccines such as Intervet's Porsilis® Pesti for Classical Swine Fever, and porcine circovirus 2 vaccines Circumvent® PCV and Porcilis® PCV.

1.2 Baculovirus as a vector for gene delivery into mammalian cells.

1.2.1 Discovery of *Ac*MNPV transduction of mammalian cells.

Volkman and Goldsmith (1983) first showed that *Ac*MNPV can be taken up efficiently by non-target vertebrate cells such as human lung carcinoma cell line A427. Carbonell *et al*. (1985) observed that *Ac*MNPV can enter mouse L929 cells and mediate very low-level expression of *Escherichia coli* chloramphenicol acetyltransferase (CAT) gene from a Rous sarcoma virus (RSV) promoter. In the mid 1990s, two groups demonstrated that recombinant *Ac*MNPV containing mammalian cell-active promoters could transduce and express foreign genes in mammalian cells. Recombinant *Ac*MNPV containing a cytomegalovirus (CMV) promoter luciferase cassette (Hofmann *et al.,* 1995) or a RSV long terminal repeat (LTR) promoter *lac*-Z cassette (Boyce and Bucher, 1996) were observed to cause high levels of reporter gene expression in both primary hepatocytes and hepatoma cells. Shoji *et al*. (1997) demonstrated that *Ac*MNPV carrying *β*-galactosidase (*β* -gal) under the control of a stronger promoter CAG (cytomegalovirus enhancer, chicken *β*-actin promoter, and rabbit β-globin poly(A) signal) can be used to express *β* -gal in a variety of mammalian cell lines. Since the discovery that *Ac*MNPV can efficiently transduce certain mammalian cell lines, this virus has become a potential vector for gene delivery into mammalian cells such as cell-based drug screening (Condreay *et al*., 2006).

1.2.2 Susceptible cell lines.

Since the study by Volkman and Goldsmith (1983), a variety of non-host cell lines have

been evaluated for *Ac*MNPV transduction and the list of cell lines transduced by *Ac*MNPV has expanded rapidly (reviewed by Hu, 2006). A list of cell lines reported to be susceptible to *Ac*MNPV transduction is shown in Table 1.1. Cell lines transduced by *Ac*MNPV include human cells (e.g., HeLa, HepG2), non-human primate cells (e.g., COS-7, CV-1), rabbit cells (e.g., primary hepatocytes), rodent cells (e.g., CHO, BHK), porcine cells (e.g., CPK, FS-L3), bovine cells (e.g., MDBK, BT), fish cells (EPC, CHSE-214), chicken primary myoblast cells and whole embryonic fibroblast cells. Efficient transduction of various neuroblastomal, nonneuronal cell lines, as well as three human neural primary cultures has also been observed (Sarkis *et al.,* 2000). Moreover, it has been demonstrated that mesenchymal stem cells (MSC) from human umbilical cord blood and bone marrow, MSC-derived adipogenic, osteogenic, and chondrogenic progenitor cells can also be transduced by *Ac*MNPV (Ho *et al.,* 2005; 2006).

1.2.3 Factor that affect transduction efficiency.

Previous studies showed that transduction efficiencies could range from greater than 90% for BHK cells when transduced with a recombinant *Ac*MNPV carrying a CMV-eGFP cassette (Chiang *et al.,* 2006) to less than 10 % for NIH-3T3 cells when transducing with another recombinant *Ac*MNPV with a CMV-eGFP cassette. This variability is not only cell line dependent, but is also dependent on the transduction temperature, duration, media as well as the promoter used to drive the reporter gene.

A. Cell type:

Early data suggested that *Ac*MNPV can efficiently transduce hepatocytes (e.g., HepG2,

Huh-7) from different sources (e.g., human and rabbit). The efficiencies of hepatocyte transformation can be as high as 80% (Wang *et al.,* 2005). The cellular differentiation state may also be a factor influencing transduction efficiency. Sarkis *et al*. (2000) reported a transduction efficiency of only 30% for undifferentiated human neural progenitor cells, but for differentiated neural cells, the efficiency increased to 55%. Despite the observation that many kinds of cell lines can be efficiently transduced by *Ac*MNPV, certain cell lines of hematopoietic origin, such as U937, K562, Raw264.7 (Condreay *et al.,* 1999), LCL-cm, and Raji (Cheng *et al.,* 2004), are not transduced efficiently.

B. Transduction temperature, duration, and media used:

Hsu *et al.* (2004) reported a more efficient transduction protocol than previously used protocols. Recombinant AcMNPV with a CMV-eGFP reporter gene (Bac-CE) had higher transduction efficiency at 25°C (78%) than at 37°C (56%) and 4°C (45%). The maximum transduction efficiency of Bac-CE on HeLa cells was reached after 4 hr incubation. Extending the duration of transduction to 8 hr did not significant increase the efficiency. The authors also noted that using PBS as a transduction medium gave the highest efficiency for HeLa, HepG2, Huh7, and chondrocytes, while using TMN-FH (Sf9 insect cell medium) or DMEM (mammalian cell medium) reduced the transduction efficiency by approximately 10% and 60%, respectively. Transient gene expression in mammalian cell lines (eg, HeLa, CHO, COS-7, 293, primary human keratinocytes and bone marrow fibroblasts) can be enhanced by adding a selective histone deacetylase inhibitor Trichostatin A (TSA) or sodium butyrate (Condreay *et al.,* 1999).

C. Promoter:

Most transduction studies have relied on reporter gene cassettes that consist of either GFP or luciferase under the control of a mammalian cell active promoter. The promoters used to drive the reporter gene can have a significant impact on the expression level of reporter genes in mammalian cells and can impact the observed transduction efficiency. Shoji *et al*. (1997) compared the expression level of luciferase under the control of either a CAG promoter or a CMV reporter. *Ac*MNPV carrying either a CAG promoter-luciferase or CMV promoter-luciferase cassettes both result in high levels of luciferase expression in transduced HepG2, Huh-7, and COS-7 cells. However, tenfold higher luciferase activity was observed in HeLa cells transduced with the *Ac*MNPV carrying a CAG promoter driven cassette than cells transduced with viruses carrying a CMV promoter driven cassette (Shoji *et al.,* 1997). To date, various promoters have been incorporated into *Ac*MNPV to drive reporter gene expression in mammalian cells. Spenger *et al*. (2004) compared the expression levels of GFP-luciferase fusion protein driven by Simian virus 40 (SV40), CMV, RSV and a cellular promoter (human ubiquitin C) in CHO, COS-1, and HEK293 cells. Results showed that transgene expression was highest with the CMV and RSV promoter, followed by the human ubiquitin C promoter, and lowest with the SV40 promoter (Spenger *et al.,* 2004). Recently, it was observed that white spot syndrome virus (WSSV) *ie1* promoter in the context of a recombinant *Ac*MNPV displayed strong promoter activity in both insect and mammalian cells (Gao *et al.,* 2007).

Transcriptional targeting can also be achieved by using cell/tissue-specific promoters. Park *et al*. (2001) reported that recombinant *Ac*MNPV carrying the luciferase gene under

the control of human alpha-fetoprotein (AFP) promoter/enhancer expressed luciferase in AFP-producing Huh7, Hep3B, and HepG2 cell lines, but not in AFP-nonproducing cell lines. It has also been observed that recombinant *Ac*MNPV carrying a human *fms*-like tyrosine kinase-1 promoter (flt-1-GFP cassette) was able to target GFP expression specifically to retinal vasculature in rat eyes *in vivo* (Luz-Madrigal *et al.,* 2007).

1.2.4 Gene delivery *in vivo.*

Use of recombinant *Ac*MNPV as a tool for gene delivery *in vitro,* as well as *in vivo,* has been studied intensively (reviewd by Hu, 2006). Gene delivery *in vivo* using recombinant *Ac*MNPV is complicated by the fact that the virus is rapidly inactivated by serum complement (Hofmann and Strauss, 1998). However, protection of *Ac*MNPV-vectors against complement-mediated inactivation can be achieved by adding recombinant soluble complement receptor type 1 (sCR1) to the virus (Hofmann *et al.,* 1998) or by fusion of human decay-accelerating factor (DAF) to GP64 envelope protein to form a DAF-GP64 fusion protein that is displayed on the envelope of *Ac*MNPV (Huser *et al.,* 2001). Vesicular stomatitis virus G protein (VSV-G)-pseudotyped *Ac*MNPV increased the transduction efficiency of skeletal muscle, brain and testes in a mouse model when compared with the transduction efficiency of control viruses lacking VSV-G (Pieroni *et al.,* 2001; Tani *et al.,* 2003). These results suggest that *Ac*MNPV pseudotyped with VSV-G are more resistant to complement-inactivation. Thus, genetic engineering of the viral surface can be an important strategy for improving *Ac*MNPV as a gene delivery vector for *in vivo* applications (Mottershead *et al.,* 1997, 2000; Grabherr *et al.,* 2001; Ojala 2001; Oker-Blom *et al.,* 2003; Raty *et al.,* 2004; Riikonen *et al.,* 2005). A summary

of published studies using *Ac*MNPV for *in vivo* gene delivery is presented in Table 1.2.

1.3 Viral envelope fusion proteins.

1.3.1 Group I NPV envelope fusion protein - GP64.

Surface glycoproteins of viruses play a very important role in the control and regulation of cellular recognition and virus entry. GP64 is the major envelope protein of *Ac*MNPV BV and it is required for binding to cell surface receptors during viral entry by receptor mediated endocytosis (Hefferon *et al.,* 1999). Once the virus is in the endosome, GP64 also mediates low-pH dependent membrane fusion of the viral envelope with the endosome membrane and leads to release of the viral nucleocapsids (NCs) into the cytoplasm (Blissard and Wenz, 1992). GP64 is also essential for virion budding from the cell surface during viral egress (Oomens and Blissard, 1999). Previous studies indicated that GP64 is essential for virus propagation both in animals and in tissue culture (Oomen, 1999; Lung *et al.,* 2002), as well as for the spreading of viral infection from cell to cell (Monsma *et al.,* 1996).

To address the role GP64 plays in *Ac*MNPV transduction of mammalian cells, Tani *et al*. (2001) demonstrated that a recombinant *Ac*MNPV with an extra copy of the *gp64* gene can incorporate about 1.5 times the normal amount of GP64 on the virion surface and exhibit 10- to 100-fold more luciferase expression (under the control of the CAG promoter) in a variety of mammalian cell lines when compared to control *Ac*MNPV with a single copy of the *gp64* gene. Liang *et al*. (2005) have further demonstrated that while

Helicoverpa armigera nucleopolyhedrovirus (*Hear*NPV, a group II NPV, and thus has no *gp64* gene) does not transduce BHK, HepG2, HeLa, Vero and PK-15 cells. However, *Hear*NPV pseudotyped with *Ac*MNPV GP64 (adding GP64 to the surface of *Hear*NPV virions) has the same tropism as *Ac*MNPV and can transduce all of the mammalian cell lines tested. These results suggest that GP64 plays an important role in the interaction of *Ac*MNPV with mammalian cells.

1.3.2 Group II NPV envelope fusion protein – F protein.

A different type of envelope fusion protein (F protein) has been identified in group II NPVs - viruses lacking the *gp64* gene (IJkel *et al.,* 2000, Pearson *et al.,* 2000). F proteins from group II NPVs such as *Spotoptera exigua* multiple nucleopolyhedrovirus (*Se*MNPV) and *Lymantria dispar* multiple nucleopolyhedrovirus (*Ld*MNPV) have low pH-dependent fusion activity (IJkel *et al.,* 2000; Pearson *et al.,* 2000). The *Se*MNPV F protein is synthesized as a proprotein (F_0) that is cleaved into two subunits, F_1 and F_2 (IJkel *et al.,* 2000). The cleavage of the precursor protein (F_0, 80-kDa) into a small N-terminal fragment (F_2, 21-kDa) and a large C-terminal fragment (F_1, 60-kDa) is required for activation of the *Se*MNPV F protein, and is mediated by a furin-like proprotein convertase (Westenberg, 2002). Recently, it was shown that an *f*-null *Helicoverpa armigera* NPV (*Hear*NPV) could not produce infectious BV when transfected into HzAM1 cells, indicating the *Hear*F protein is essential for virus entry and egress (Wang *et al.,* 2008). Previous data showed that F proteins from group II NPVs are functional analogs of GP64 and that both the *Ld*MNPV *f* gene and the *Se*MNPV *f* gene can rescue a *gp64*-null *Ac*MNPV and result in production of infectious viral particles (Lung *et al.,*

2002). However, a *Se*F-null *Se*MNPV could not be rescued by insertion of the *Ac*MNPV *gp64* gene, indicating the functional analogy between GP64 and F is not reciprocal (Westenberg and Vlak, 2008). This is likely due to incompatible interactions between the fusion proteins and proteins in the heterologous virus or with cellular factors. The much longer cytoplasmic tail domain (CTD) of F protein - in comparison with GP64 - found in *Se*MNPV and other group II NPVs may be required for interaction with nucleocapsids to promote budding of group II NPVs, whereas this interaction might not be needed for the production of *Ac*MNPV and other group I NPV BVs (Long *et al.,* 2006).

F homologues have also been found in group I NPVs, such as *Ac*MNPV which do have a *gp64* gene. The F homologue of *Ac*MNPV (*Ac23*), is localized to the BV envelope, but has no detectable membrane fusion activity. *Ac23* is also dispensable for *Ac*MNPV infectivity in both animals and cultured cells (Lung *et al.,* 2003). However, *Ac23* is a pathogenicity factor that accelerates host death by approximately 28% or 26 hours (Lung *et al.,* 2003), indicating that *Ac23* may be involved in other functions that enhance viral infection (for details see chapter 3).

1.3.3 Modification of virus envelope protein (pseudotyping).

The tropism and the transduction efficiency of *Ac*MNPV on non-host cells could potentially be modulated by: 1) fusing targeting molecules (peptides or antibodies) to the major *Ac*MNPV envelope protein, GP64, and displaying the fused targeting molecule on the virion surface (Tami *et al.,* 2000), or 2) displaying glycoproteins from heterologous viruses on the *Ac*MNPV envelope, either in place of, or in addition to the endogenous

GP64 envelope protein (Barsoum *et al.,* 1997; Mangor *et al.,* 2001; Park *et al.,* 2001; Pieroni *et al.,* 2001; Tani *et al.,* 2001; 2003; Lung *et al.,* 2002; Facabene *et al.,* 2004; Kitagawa *et al.,* 2005). Barsoum *et al*. (1997) reported that VSV-G expressed with the polyhedrin promoter is incorporated into *Ac*MNPV budded virion. These VSV-G-pseudotyped *Ac*MNPV had a tenfold augmented expression of a *lacZ* reporter gene (under the control of RSV-LTR promoter) in HepG2 cells when compared with *Ac*MNPV without VSV-G. Park *et al*. (2001) similarly demonstrated that a VSV-G-pseudotyped *Ac*MNPV carrying a luciferase reporter gene under the control of human alpha-fetoprotein (AFP) promoter transduced human hepatoma cells *in vitro* with fivefold greater efficiency than *Ac*MNPV lacking VSV-G. Both these reports used viruses that contained both VSV-G and the endogenous envelope protein GP64.

In addition to VSV-G, F protein from *Se*MNPV (*Se*F) and *Ld*MNPV (*Ld*F) have also been used to pseudotype *gp64null-Ac*MNPV, in which the endogenous GP64 protein is deleted (Mangor *et al.,* 2001; Lung *et al.,* 2002; Kitagawa *et al.,* 2005). VSV-G, *Se*F, and *Ld*F pseudotyped *gp64null-Ac*MNPV have delayed infection kinetics and have reduced viral titer in comparison to *wild-type Ac*MNPV. Transduction of human 293T and hamster BHK cells with the VSV-G-pseudotyped *gp64null-Ac*MNPV showed that expression of luciferase reporter gene (under the control of CAG promoter) was dose-dependent and the expression level was similar to a control virus without VSV-G (Kitagawa *et al.,* 2005). However, expression of a GFP reporter gene under the control of CMV promoter were not detectable when BHK-21, LLC-PK1 and H35 cells were transduced with *Se*F-pseudotyped *gp64null-Ac*MNPV (Westenberg *et al.,* 2007), suggesting that *Se*F protein

receptors are not present on these mammalian cells.

To date, the cell surface receptor molecule(s) that mediate *Ac*MNPV binding and entry to insect and non-insect cells have not been identified. However, ligand-directed gene delivery has been elegantly demonstrated by Kitagawa et al. (2005). Both CD46 and SLAM (receptors for measles virus) can be incorporated onto the surface of *gp64null-Ac*MNPV, and the small amount of virions generated will specifically transduce BHK cells that have been engineered to co-express EdH and EdF - glycoproteins on the Edmonston strain of the measles virus - on the cell surface. Additionally, they demonstrated that CD46-pseudotyped *gp64null-Ac*MNPV did not transduce cells co-expressing IcH and IcF, glycoproteins found on the Ichinose strain of measles virus that do not use CD46 as receptors, whereas SLAM-pseudotyped *gp64null-Ac*MNPV could. These results indicate that pseudotyped *Ac*MNPV displaying targeting molecules can be used for targeted gene delivery. However, a major drawback is that these pseudotyped gp64-null *Ac*MNPV are unable to be amplified due to the lack of an essential GP64 gene, and therefore have very limited practical use.

1.4 Summary

There are many properties that make *Ac*MNPV superior to vectors based on mammalian viruses for gene delivery into mammalian cells. *Ac*MNPV replication is restricted to certain insect cells, so it is not cytotoxic to mammalian cells; mammals initially do not have antibodies against this insect virus; *Ac*MNPV has a large capacity for foreign DNA

(estimated capacity for extra DNA is more than 38kb); *Ac*MNPV can enter many mammalian cell lines and thus can be used for delivery and expression of genes in these mammalian cells. An introduction to baculovirus and its use in biotechnology related application is presented in **Chapter 1**. The broad tropism of *Ac*MNPV for a variety of cell types is desirable for certain types of *in vitro* applications, but is undesirable for *in vivo* gene delivery/therapy that requires targeting of specific cell types or tissues. A new method for generating pseudotyped *Ac*MNPV with narrower tropism for mammalian cells will be described in **Chapter 2**. Ac23 is a fusion (F) protein homologue that has no detectable fusion activity, and is dispensable for *Ac*MNPV infectivity in both animals and culture cells (Lung *et al.,* 2003). Previous studies showed that *Ac23* is a pathogenicity factor that accelerates host death, the mechanism by which this is achieved is not known. A study of *Ac23* function and how *Ac23* contributes to hasten host death will be discussed in **Chapter 3**.

Table 1.1 Cells susceptible to transduction by *Ac*MNPV.

Cell lines	**Reference**
Human cells HepG2	Boyce and Bucher, 1996; Hofmann *et al.,* 1995; Tani 2001; Mahonen *et al.,* 2007; Gao *et al.,* 2007; Shoji *et al.,* 1997; Wang *et al.,* 2004
Huh-7	Boyce and Bucher, 1996; Condreay *et al.,* 1999; Shoji *et al.,* 1997; Wang *et al.,* 2004
HeLa	Boyce and Bucher, 1996; Condreay *et al.,* 1999; Chan *et al.,* 2005; Ge *et al.,* 2007; Gao *et al.,* 2007; Shoji *et al.,* 1997; Wang *et al.,* 2004
Raji	Ge *et al.,* 2007
HR1	Ge *et al.,* 2007
BJAB	Ge *et al.,* 2007
293	Sollerbrant *et al.,* 2001; Liang *et al.,* 2004; Kitagawa *et al.,* 2005; Chan *et al.,* 2005; Kenoutis *et al.,* 2006; Ge *et al.,* 2007; Mahonen *et al.,* 2007; Gao *et al.,* 2007
WI38	Condreay *et al.,* 1999
MRC5	Palombo *et al.,* 1998; Yap *et al.,* 1998
MG63	Condreay *et al.,* 1999
ECV-304	Airenne *et al.,* 2000
HUVEC	Kronschnabl *et al.,* 2002
PC3	Stanbridge *et al.,* 2003
KATO-III	Shoji *et al.,* 1997
WISH	Changyong *et al.,* 2006
D98-HR1	Ge *et al.,* 2007
Osteosarcoma SAOS-2	Condreay *et al.,* 1999; Song *et al.,* 2003
Pancreatic β cells	Ma *et al.,* 2000

Keratinocytes	Condreay *et al.,* 1999
Bone marrow fibroblast	Condreay *et al.,* 1999
Primary foreskin fibroblasts	Dwarakanath *et al.,* 2001
Primary neural cells	Sarkis *et al.,* 2000
SMMC-7721(hepatoma)	Changyong *et al.,* 2006
Nonhuman primate cells COS-7	Condreay *et al.,* 1999; Liang *et al.,* 2004; Shoji *et al.,* 1997
Vero	Poomputsa *et al.,* 2003; Airenne, *et al.,* 2000; Changyong *et al.,* 2006; Gao *et al.,* 2007
CV-1	Tani *et al.,* 2001
B95-8	Ge *et al.,* 2007
Rodent cells CHO	Condreay *et al.,* 1999; Hu *et al.,* 2003a; Chan *et al.,* 2005
BHK	Condreay *et al.,* 1999; Hu *et al.,* 2003a; Tani *et al.,* 2001; Liang *et al.,* 2004; Kitagawa *et al.,* 2005; Chan *et al.,* 2005; Changyong *et al.,* 2006; Chiang *et al.,* 2006; Westenberg *et al.,* 2007; Gao *et al.,* 2007; Wang *et al.,* 2004
RGM1	Shoji *et al.,* 1997
PC12	Shoji *et al.,* 1997
N2a	Sarkis *et al.,* 2000
L929	Airenne *et al.,* 2000; Cheng *et al.,* 2004; Changyong *et al.,* 2006
H35	Westenberg *et al.,* 2007
Primary mouse kidney cells (MKC)	Liang *et al.,* 2004
Mouse Pancreatic β cells	Ma *et al.,* 2000
Rat hepatic stellate cells	Gao *et al.,* 2002
Primaryosteoblast and osteoclast	Boyce and Bucher, 1996

Primary rat hepatocytes	Tani *et al.,* 2003
Rat articular chondrocyte	Ho *et al.,* 2004; Wang *et al.,* 2004
Rabbit cells Rabbit aortic smooth muscle	Raty *et al.,* 2004; Mahonen *et al.,* 2007
RK13	Nakamichi *et al.,* 2002
Primary hepatocytes	Munger and Roizman, 2001
Bovine cells MDBK	Aoki et a., 1999; Airenne, K.J. 2000
BT	Aoki *et al.,* 1999
Porcine cells CPK	Aoki *et al.,* 1999; Shoji *et al.,* 1997
FS-L3	Shoji *et al.,* 1997
PK-15	Aoki *et al.,* 1999; Airenne, *et al.,* 2000; Liang *et al.,* 2004; Gao *et al.,* 2007
LLC-PK1	Westenberg *et al.,* 2007
Fish cells EPC	Leisy *et al.,* 2003
CHES-214	Leisy *et al.,* 2003
CHH-1	Leisy *et al.,* 2003
Embryo	Wagle and Jesuthasan, 2003
Avian chicken primary myoblast cells	Ping *et al.,* 2006
Embryonic fibroblast cells	Ping *et al.,* 2006
Primary liver cells	Song *et al.,* 2006
Primary kidney cells	Song *et al.,* 2006
Primary lung cells	Song *et al.,* 2006

Table 1.2 *In vivo* studies of *Ac*MNPV-mediated transduction.

Envelope protein	Trans/Reporter gene	Target organ	Delivery method	Reference
GP64	GFP	Rodent brain	Direct injection into brain	Sarkis *et al.,* 2000
	lacZ	Rat brain	Direct injection into brain	Lehtolainen *et al.,* 2002
	GFP	Rat eye	Direct injection into vitreous body	Luz-Madrigal *et al.,* 2007
	luciferase	Rat brain	Stereotaxic injection into the rat striatum	Li *et al.,* 2004
	lacZ	Mouse liver	Portal vein infusion	Hoare *et al.,* 2005
	lacZ	Rabbit carotid arteries	A silastic collar placed around rabbit carotid arteries	Airenne *et al.,* 2000
	Pseudorabies virus glycoprotein gB	Muscle, nose	Direct injection into muscle	Aoki *et al.,* 1999
GP64 + soluble DAF*	human factor IX (hFIX)	Rat liver	Direct injection into liver	Huser *et al.,* 2001
GP64 + VSV-G	*lacZ*	Mouse skeletal muscle	Direct injection into mouse quadriceps	Pieroni *et al.,* 2001
	lacZ	Mouse striatum	Direct injection into the mouse striatum	Kobayashi *et al.,* 2006
	GFP	Mouse brain, testes	Direct injection into mouse brain	Tani *et al.,* 2003

*DAF: human decay-accelerating factor

Chapter 2 – Pseudotyping *gp64*null-*Ac*MNPV with the F protein from *Se*MNPV diminishes transduction efficiency into non-insect cells.

2.1 Introduction.

Baculovirus are insect viruses with many biotechnological and biomedical applications. Recently it was discovered that the type baculovirus, *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV), can efficiently enter a broad range of mammalian cell lines including human HepG2 (Tani *et al.,* 2001), monkey VERO (Airenne *et al.,* 2000), murine BHK-21 (Condreay *et al.,* 1999), porcine PK-15 (Airenne *et al.,* 2000) as well as primary cells such as human primary hepatocytes (Hofmann *et al.,* 1995), rat primary hepatocytes (Boyce and Bucher, 1996), chicken primary myoblast cells and whole embryonic fibroblast cells (Ping *et al.,* 2006). Upon entry into non-insect cells *Ac*MNPV does not replicate, and appear to cause little or no cytopathic effect. Thus, *Ac*MNPV is a useful tool for delivering genes into mammalian cell lines for applications such as cell-based drug screening (reviewed by Kost *et al.,* 2005). The use of *Ac*MNPV as a vector for delivering genes into mammalian cells *in vivo*, for applications such as gene therapy or as a vector for vaccine delivery is also being explored (reviewed by van Oers, 2006). Advantages of using *Ac*MNPV as a vector for gene delivery into mammalian cells *in vivo* include: 1) a large capacity for foreign DNA; the viral genome can be easily engineered and propagated with an insertion of more than 38kb containing multiple genes (Cheshenko *et al.,* 2001); 2) a better biosafety profile since *Ac*MNPV does not replicate or express most of its genes in mammalian cells, and thus in comparison with other viral

vectors (e.g., Retrovirus, Lentivirus, Adenovirus) that could cause serious toxicity or induce oncogenesis in human cells, there is little observable cytopathic effect (reviewed by Hu, 2006); 3) antibodies against this insect virus are initially absent in humans (Van Oers, 2006). One of the obstacles for *Ac*MNPV-mediated *in vivo* gene delivery is complement-mediated inactivation of *Ac*MNPV (Hofmann and Strauss, 1998). Protection of *Ac*MNPV-vectors from complement-mediated inactivation have been observed with recombinant soluble complement receptor type 1 (sCR1, Hofmann *et al.,* 1998), and also by incorporation of decay-accelerating factor (DAF) into the *Ac*MNPV envelope (Huser *et al.,* 2001). Studies also showed that a recombinant *Ac*MNPV displaying or pseudotyped with the envelope protein of a heterologous virus, vesicular stomatitis virus G protein (VSV-G) could efficiently transduce cells in the mouse skeletal muscle, cerebral cortex and testis *in vivo* (Pieroni *et al.,* 2001; Tani *et al*. 2003), suggesting that *Ac*MNPV modified with VSV-G protein might have the ability to resist complement inactivation. Thus, modification of viral surface proteins is a potentially useful approach for improving the use of *Ac*MNPV for *in vitro* and *in vivo* gene delivery (Grabherra*,* 2001; Mottershead, *et al.,* 1997, 2000; Ojala, 2001; Oker-Blom, *et al.,* 2003; Raty, *et al.,* 2004; Riikonen, *et al.,* 2005).

The ability of *Ac*MNPV to enter many types of mammalian cells has been a desirable trait for its use in applications such as cell-based drug screening assays. However, this broad tropism may be less desirable for *in vivo* applications that require or benefit from gene delivery into specific cell types. Virus binding and entry into non-target cell types could dilute the input virus, decrease the efficacy, and have negative consequences on

unintended target cells. Therefore, finding ways to generate viral vectors that can target specific cell types is an important step in advancing the use of *Ac*MNPV for *in vivo* applications. *Ac*MNPV budded virus (BV) enter host insect cells via endocytosis, mediated by the major BV envelope protein GP64 (Blissard and Wenz, 1992). Previous studies have shown that phospholipids on the cell surface might be important docking spots for the GP64 protein (Tani *et al.,* 2001), but the receptor for *Ac*MNPV in insect cells has not been identified. Viral tropism could be modulated by altering proteins on the surface of the virion (pseudotyping). Pseudotyping *Ac*MNPV with VSV-G has been done in the presence or absence of the endogenous envelope protein GP64 (Barsoum *et al.,* 1997; Mangor *et al.,* 2001). Kitagawa *et al*. (2005) showed that expression of the luciferase reporter gene in 293T cells transduced with VSV-G pseudotyped *gp64null*-*Ac*MNPV was inhibited by antibodies against VSV-G. This result suggests that VSV-G pseudotyped *gp64*null *Ac*MNPV-mediated gene expression in the 293T cells is dependent on interactions mediated by VSV-G proteins on the virion. Pseudotyping of *Ac*MNPV by displaying a targeting molecule on the surface of the virion has been shown to target viral entry into specific cell types. Kitagawa *et al*. (2005) elegantly showed that *gp64null*-*Ac*MNPV pseudotyped with CD46 or SLAM (receptors of measles virus) are specifically targeted to cells expressing measles virus envelope glycoproteins. However, *gp64* is an essential gene that is required for progeny virus production (Oomens *et al.,* 1999; Lung *et al.,* 2002). Deletion of *gp64* results in extremely low yield of *gp64*-minus viruses that can not be propagated and amplified, thus limiting the practical use of these types of viruses. Previous results have shown that envelope fusion proteins from heterologous viruses such as *Se*MNPV or *Ld*MNPV (*Se*F and *Ld*F) can also functionally substitute for

GP64 and result in the production of significantly higher amounts of *gp64*-minus progeny viruses that are easily propagated (Lung *et al.,* 2002). The *gp64*-minus *Ac*MNPV viruses display *Se*F protein on its surface and are called *Se*F-pseudotyped *gp64*-null *Ac*MNPV (Lung *et al.,* 2002). Several lines of evidence suggest that *Se*F will likely not confer *Se*F-pseudotyped *gp64null-Ac*MNPV with the ability to transduce mammalian cells efficiently. To determine whether *Ac*MNPV tropism for non-insect cells can be narrowed by pseudotyping *gp64*-null *Ac*MNPV with *Se*F, a recombinant *Se*F-pseudotyped *gp64*-null *Ac*MNPV virus expressing the humanized renilla green fluorescent protein (hrGFP) under the control of a cytomegalovirus (CMV) promoter was generated. The transduction efficiency of the *Se*F-pseudotyped *gp64*-null *Ac*MNPV virus was determined on 15 cell lines derived from human, monkey, murine, porcine, feline, canine, bovine, ovine, avian, and fish species. Here, I show that *Se*F-pseudotyped *gp64null Ac*MNPV has very low or no detectable reporter gene expression in non-insect cells, suggesting this pseudotyped virus could potentially serve as a platform for generating "designer viruses" for targeted gene delivery *in vivo*.

2.2 Materials and Methods

2.2.1 Cloning and plasmid construction.

2.2.1.1 PCR and primers.

All PCRs were performed with GeneAmp® PCR system 9700 thermocycler (Applied Biosystems) and Phusion™ High-Fidelity DNA polymerase (Fermentas) according to the manufacturer's instructions. The typical reaction volume was 50 µL (10µL Phusion HF

buffer (5X), 1μL dNTPs (10mM), 5 μL primers (5 μM), 1 μL DNA template(10ng total), 0.5 μL Phusion DNA polymerase, and 27.5 μL H_2O) and a typical cycle program is 1 cycle at 98°C (30 sec), followed by 27 cycles of 98°C (10 sec), 50-60°C (30 sec), 72°C (30 sec/1 kb), and a final extension of 7 min at 72°C. The primers used for PCR amplification are summarized in Table 2.1.

2.2.1.2 Cloning of reporter genes for detection of expression in non-insect cells.

The open reading frames (ORF) of luciferase (Luc) and hrGFP were amplified using the plasmid pCMV-Luc (Dr. Yu-Chan Chao, Academia Sinica, Taiwan) and plasmid pIRES-hrGFP-1a (Stratagene) as template, respectively. Primers used to amplify Luc (P5'*Sac*I-Luc and P3'Luc-*Xho*I) were designed to generate *Xho*I and *Sac*I restriction sites upstream and downstream of the Luc ORF, respectively. Primers for hrGFP amplification (P5'-*Sac*I-hrGFP and P3'hrGFP-*SexA*I) were designed to generate *Sac*I and *SexA*I restriction sites upstream and downstream of the hrGFP ORF, respectively.

The PCR products were digested with restriction enzymes and cloned downstream of the CMV promoter in the plasmid pIRES-hrGFP-1a Δ 3x FLAG-IRES-hrGFP. The resulting constructs were named pCMV-Luc (KY1) and pCMV-hrGFP (KY4). Luc and hrGFP ORF under the control of a CMV promoter were re-amplified by PCR using KY1 and KY4 as templates and cloned into a shuttle vector pFBDM (Berger *et al.,* 2004), which contains a Tn*7* transposon harbouring baculovirus promoter upstream of multiple cloning sites, to generate pFB/CMV-Luc (KY3) and pFB/CMV-hrGFP (KY5), respectively. Primers P5'NruI-CMV and P3'Luc-XbaI were used to amplify the CMV-Luc fragment,

while primers P5'NruI-CMV and P3'-hrGFP-XbaI were used to amplify the CMV-hrGFP fragment.

2.2.1.3 Cloning of envelope protein genes and insect cell reporter genes.

A total of three PCR amplified fragments containing a β-glucuronidase (GUS) reporter gene (under the control of the P6.9 promoter) with or without genes encoding the envelope proteins GP64 and *Se*F, under the control of the gp64 promoter were amplified. Plasmids pΔFB/gus(R)*Ac*gp64-10dATG (Lung *et al*., 2002) were used as the template for the *gp64* containing fragment (P6.9-GUS-Pgp64-GP64) and pΔFB/gus(R)Se8-32+stop-ATG (Lung *et al*., 2002) were used as template for both the *Se*F containing fragment (P6.9-GUS-Pgp64-*Se*F) and the fragment without envelope protein genes (P6.9-GUS). P6.9-GUS was amplified using primers P5'-AvrII-P6.9 and P3'gus-RsrII-I-Scel-Cfr9I. P6.9-GUS-Pgp64-*Se*F was amplified using primers P5'-AvrII-P6.9 and P3'*Se*F-RsrII-I-Scel-Cfr9I. P6.9-GUS-Pgp64-GP64 was amplified using primers P5'-AvrII-P6.9 and P3'GP64-RsrII-I-Scel-Cfr9I. PCR products were cloned into pJET1/blunt cloning vector according to the manufacturer's instructions (Fermentas) to generate pJET/P6.9-GUS (KY7), pJET/P6.9-GUS-Pgp64-*Se*F (KY8), and pJET/P6.9-GUS-Pgp64-GP64 (KY9). Each of the three vectors were double digested with *Avr*II and *Cfr9*I, and gene fragments containing the envelope protein gene and/or the GUS gene were gel purified and cloned individually into the two shuttle vectors KY3 and KY5. Thus, a total of six shuttle vector constructs with different combination of genes were generated: pΔFB/P6.9-GUS-CMV-Luc (KY13), pΔFB/P6.9-GUS-Pgp64-*Se*F-CMV-Luc (KY14), pΔFB/P6.9-GUS-Pgp64-GP64-CMV-Luc (KY15), pΔFB/P6.9-GUS-CMV-hrGFP (KY16), pΔFB/P6.9-GUS-

Pgp64-*Se*F-CMV-hrGFP (KY17), pΔFB/P6.9-GUS-Pgp64-GP64-CMV-hrGFP (KY18). A summary of the plasmid constructs generated for this work is presented in Table 2.2.

2.2.2 Cell lines and media.

Baby hamster kidney (BHK-21), human hepatocellular carcinoma (Hep G2), chicken UMNSAH/DF-1, and chicken SL-29 cells were obtained from American Type Culture Collection (ATCC). Swine Testicle (ST), Mouse McCOY, African Green monkey Kidney (VERO), Fish Stripped Snake-Head (SSN-1), Fish Gonad Rainbow trout (RTG-2), and canine MDCK cells were obtained from Dr. John Robinson (Animal Health Center, Abbotsford, BC). Bovine MDBK, fish EPC, fish CHSE-214, porcine PK-15, and ovine SCP cells were from the Canadian Food Inspection Agency, Lethbridge Laboratory, Lethbridge, AB. Sf9 cells were cultured in TMN-FH (Grace's media (Sigma) supplemented with 10% v/v FBS, 3.7g/ml Lactalbumin hydrosylate, 3.7g/ml Yeastolate, 0.35g/ml Sodium bicarbonate, pH 6.2) at 27°C. All mammalian cell lines were kept in 5% CO_2, at 37°C; and chicken UMNSAH/DF-1 cells were kept in 5% CO_2 at 39°C. HepG2, PK-15 and BHK-21 cells were maintained in Minimum Essential Medium (MEM, Hyclone) supplemented with 1% (v/v) sodium pyruvate and 10% (v/v) defined Fetal Bovine Serum (DFBS, Hyclone) at 37°C, 5% CO_2. CHSE-214 and EPC cells were mantained in MEM supplemented with 10% (v/v) characterized FBS (CFBS, Hyclone) at room temperature. ST, McCOY, SSN-1, and RTG-2 cells were cultivated in Eagle's MEM (Sigma) with 10% DFBS. ST was also supplemented with 1% non-essential amino acid (Sigma). VERO cells were maintained in Eagle's Basal Medium (Sigma) supplemented with 5% DFBS, MDCK cells were maintained in Alpha MEM

supplemented with 10% DFBS, and UMNSAH/DF-1 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, ATCC) supplemented with 10% DFBS. Cells, media and culture conditions are summarized in Table 2.3.

2.2.3 Generation of recombinant virus.

Recombinant viruses were generated as described in the Bac-to- Bac ® baculovirus Expression System manual (Invitrogen). Six recombinant shuttle vectors with the Luc or hrGFP reporter gene (KY13-KY18) were transformed into DH10*gp64null*Bac electro-competent *E. coli* cells harboring the *gp64null* bacmid, *gp64null*-pMON14272 (Lung *et al.,* 2002) and the helper plasmid pMON7124 which contains the Tn*7* transposase gene necessary for Tn*7*-mediated transposition of genes on the shuttle plasmid onto the bacmid. The resulting recombinant bacmids were named b*gp64nullAc*Bac/ΔFBgus-CMV-hrGFP, and b*gp64nullAc*Bac/ΔFBgus-CMV-Luc (no envelope protein gene transposition); b*gp64nullAc*Bac/ΔFBgus-*Se*F-CMV-hrGFP and b*gp64nullAc*Bac/ΔFBgus-*Se*F-CMV-Luc (*Se*F gene transposition); b*gp64nullAc*Bac/ΔFBgus-GP64-CMV-hrGFP and b*gp64nullAc*Bac/ΔFBgus-GP64-CMV-Luc (*gp64* gene transposition), respectively. Four of the six shuttle vectors (KY 13, 14, 16 and 17) were also transformed into *E. coli* DH10Bac electrocompetent cells harboring the helper plasmid, pMON7124 and the *Ac*MNPV bacmid pMON14272 with the *gp64* envelope protein gene intact. The resulting recombinant bacmids were called b*Ac*Bac/ΔFBgus-CMV-hrGFP and b*Ac*Bac/ ΔFBgus-CMV-Luc (with one copy of the *gp64* gene); b*Ac*Bac/ΔFBgus-*Se*F-CMV-hrGFP and b*Ac*Bac/ΔFBgus-*Se*F-CMV-hrGFP (with one copy of the *gp64* gene and one copy of the *Se*F gene). A schematic representation of all recombinant bacmid constructs are

shown in Fig 2.2. The recombinant bacmids were confirmed by PCR amplification of the envelope protein genes and the reporter genes (see section 2.4). The following five of the ten recombinant bacmids carrying hrGFP reporter genes were selected for further research: b*gp64nullAc*Bac/ΔFBgus-CMV-hrGFP, b*gp64nullAc*Bac/ΔFBgus-*Se*F-CMV-hrGFP, b*gp64nullAc*Bac/ΔFBgus-GP64-CMV-hrGFP, and b*Ac*Bac/ΔFBgus-CMV-hrGFP, b*Ac*Bac/ΔFBgus-*Se*F-CMV-hrGFP. Three independent clones were initially selected for each construct, amplified overnight in 5 ml LB broth (10% w/v Tryptone, 0.5% w/v Yeast extract, 1.0% NaCl, pH 7.0) containing kanamycin (50 μg/ml), gentamycin (7 μg/ml) and tetracycline (10 μg/ml). Bacmid DNA isolation was performed according to the Bac-to-Bac® manual (Invitrogen), and 5μL of the bacmid DNA was transfected into Sf9 cells using lipofectin (Campbell, 1995). A mixture of 20μL of lipofectin (L-alpha-phosphatidylethanolamine, dioleoyl (DOPE): Dimethyldioctadecyl-ammonium bromide (DDAB) at a 2:1 molar ratio), 200μL of Grace's media (Sigma), and 5μL of bacmid DNA were incubated at room temperature for 1 hour. After the 1 hour incubation, 800μL of fresh Grace's media were added to the mixture and 1ml of the transfection solution were then added to 9 x 10^5 Sf9 cells that have been washed once with PBS. The transfected cells were incubated at 27°C for 4 hr, the transfection media was replaced with 2ml of fresh TMN-FH media, and incubated at 27 °C for another 72 hours. At 3 days post transfection (dpt, 5 dpt for b*Ac*Bac/ΔFBgus-*Se*F-CMV-hrGFP), the culture medium was harvested, centrifuged at 500 g for 5 min, and the supernatant was stored at 4°C, protected from light as the P1 virus stock. The viral titer of P1 virus stocks was determined by the end point dilution method described in O'Reilly *et al*. (1992). A single clone for each of the five viruses was chosen for analysis

and further amplified by infecting two T_{150} flasks of Sf9 cells (1.68 x 10^7cells/flask) at a multiplicity of infection (moi) of 0.1. At 3 dpi (5 days for b*Ac*Bac/ΔFBgus-*Se*F-CMV-hrGFP), medium from the infected cells was harvested and centrifuged at 500 x g for 5 min, the resulting supernatant was stored at 4°C protected from light as the P2 virus stock. P3 virus stocks were amplified from P2 virus stocks as described for the amplification of the P2 stock for v*Ac*$^{Se\text{F-CMV-hrGFP}}$, v*gp64nullAc*$^{\text{GP64-CMV-hrGFP}}$, and v*Ac*$^{\text{CMV-hrGFP}}$, respectively. The v*gp64nullAc*$^{Se\text{F-CMV-hrGFP}}$ virus was amplified using ten T_{175} flasks (1.4 x 10^7 Sf9 cells/ flask). At 3 dpi (5 dpi for v*gp64nullAc*$^{Se\text{F-CMV-hrGFP}}$), the culture medium was harvested, and centrifuged at 500 x g for 5 min. The resulting supernatant was centrifuged at 45,000 g for 1 hour at 4°C to concentrate the virus. The viral pellet was resuspended in 10 ml of the clarified supernatant plus 10 ml PBS. The resuspended concentrated virus were filtered through a 0.2 μm MFS filter (Advantec MFS), titered and store at 4°C protected from light.

2.2.4 Confirmation of constructs and virus by PCR and Western blot analysis.

Plasmids and shuttle vectors were all confirmed by PCR using gene specific primers as well as by restriction enzyme digestion. Recombinant bacmid DNAs were confirmed by three sets of primers: 1) PCR using the M13 forward and M13 reverse primers outside of Tn*7*-attachment sites was performed to ensure the Tn*7*-mediated transposition had taken place, and to rule out the presence of bacmids without an Tn*7* insert; 2) PCR using the M13 forward primer and a forward primer specific for either GUS or *gp64* or *Se*F were used to confirm the transposition of the correct envelope protein gene; 3) PCR using the M13 reverse primer and a forward primer specific to either hrGFP or Luciferase were

performed to confirm the presence of the correct reporter gene. All recombinant viruses were also confirmed by extracting DNA from virus infected Sf9 cells at 3 dpi using DNeasy Tissue Kit (Qiagen), and performing a PCR analysis using primer pairs specific to *GUS*, *Se*F and *gp64* genes. For Western blot confirmation of envelope proteins, 9 x 10^9 Sf9 cells were infected at a moi of 1, washed once in PBS, resuspended in 110 µL PBS and 110 µL Laemmli buffer (Bio-Rad) supplemented with 5% β-mercaptoethanol and boiled for 10 min. Samples were electrophoresed in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 150V for 1 hr, and transfered onto Immobilon-P membrane (Millipore) for 30 min using semi-dry transfer (Bio-Rad) at 15V. Membranes were blocked overnight in TBST (10mM Tris, 150mM NaCl, 0.05% v/v Tween-20) plus 4% skim milk at 4°C. Membranes were then incubated with either mouse anti-GP64 antibody (*Ac*V5, gift of Dr. Gary Blissard, Boyce Thompson Institute) or a chicken anti-*Se*F1 antibody (Westenberg *et al.,* 2002) at 1:1000 dilution for 1 hour at room temperature. After washing twice with TBST, membranes were incubated in AP-conjugated anti-mouse or anti-chicken 2° antibody (Medicorp) at 1:5000 dilution for 1 hour at room temperature. Blots were then washed three times with TBST and three times with TBS (5 min each). Color development using 5-bromo,4-chloro,3-indolylphosphate (BCIP)/nitroblue tetrazolium (NBT) substrate was performed as per manufacturer's protocol (Bio-Rad).

2.2.5 Transduction.

For transduction experiments, most cell lines were seeded into 24-well plates at a density of 5 x 10^4 cells/well and allowed to attach overnight. Smaller cells like EPC and SSN-1

were seeded at a density of 1 x 10^5 cells/well and bigger cells like PK-15 and BHK-21 were seeded at a density of 5 x 10^3 cells/well. Cells were then washed once with PBS, and each virus was added to duplicate wells at a moi of 500 and brought to a final volume of 200µl with PBS plus 5% (v/v) FBS. Cells were placed on an orbital shaker (Nutator, Becton Dickinson) for 6 hours at room temperature. Two ml of fresh TMN-FH growth medium were added to the cells and incubated for 24 hr at 39°C (UMNSAH/DF-1), 37°C (HepG2, BHK-21, VERO, McCOY, FKC, PK-15, MDBK, SCP, MDCK, SL-29, ST) or room temperature (EPC, CHES-214, RTG-2, SSN-1). After 24 hours of incubation, cells were fixed in 10% formalin for 3 min, washed once with PBS, and stained with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI, 300nM in PBS, Invitrogen) for 5 min. Cells were then washed twice with PBS, and photographed with a fluorescence microscope (CKX41, Olympus). Image-Pro 6.2 was used to determine the number of DAPI-stained nuclei in the micrographs, and the transduction efficiency was determined by calculating the percentage of DAPI-stained cells that were also GFP positive. To determine the infectivity of the inoculum, Sf9 cells (2.25 x 10^5 cells/ well) were infected with post-transduction inoculum, and at 3 dpi, 2 µL of X-glucuronide (X-gluc, 20mg/ml in DMSO, Biovectra) were added to each well to detect the presence of GUS activity.

Table 2.1 PCR primers used in this study.

Primer name	**Primers sequence**
P5'SacI-Luc	5'GGCCGAGCTCATGAAGCTTGGCATTCCGG-3'
P3'Luc-XhoI	5'-GGCCCTCGAGTTACAATTTGGACTTTCCGCCC-3'
P5'-SacI-hrGFP	5'-GGCCGAGCTCATGGTGAGCAAGCAGATCC-3'
P3'hrGFP-SexAI	5'-GGCCACCAGGTTTACACCCACTCGTGCAG-3'
P5'NruI-CMV	5'-GGCCTCGCGAGGCGACCGCCCAGCGAC-3'
P3'Luc-XbaI	5'GGCCTCTAGACGAGTTACAATTTGGACTTT-3'
P3'-hrGFP-XbaI	5'GGCCTCTAGATTACACCCACTCGTGCAG-3'
P5'-AvrII-P6.9	5'-AATACCTAGGCTTACGATCTGTCGACGAAATTC-3'
P3'gus-RsrII-I-Scel-Cfr9I	5'-ATATCCCGGGCGGACCGATTACCCTGTTATCCCTATCATTGTTTGCCTCCCTGCTG-3'
P3'*Se*F-RsrII-I-Scel-Cfr9I	5'-ATATCCCGGGCGGACCGATTACCCTGTTATCCCTATCACATGGGTTCCATTTCCATG-3'
P3'GP64-RsrII-I-Scel-Cfr9I	5'-ATATCCCGGGCGGACCGATTACCCTGTTATCCCTACTTAATATTGTCTATTACGGTTTC-3'
M13 forward	5'-GTTTTCCCAGTCACGAC-3'
M13 reverse	5'-CAGGAAACAGCTATGAC-3'

Table 2.2 Plasmid constructs generated in this study**.**

Construct Name	**Vector backbone**	**Inserted genes and flanking restriction enzyme sites**	***E. coli* strain transformed**
KY1	pIRES-hrGFP-1a	*Sac*I-Luc-*Xho*I	DH10B
KY3	pFBDM	*Nru*I-CMV-Luc-*Xba*I	DH10B
KY4	pIRES-hrGFP-1a	*Sac*I-hrGFP-*SexA*I	DH10B
KY5	pFBDM	*Nru*I-CMV-hrGFP-*Xba*I	DH10B
KY7	pJET1/blunt	*Avr*II-P6.9-GUS-*Rsr*II-I-*Sce*I-*Cfr*9I	DH5α
KY8	pJET1/blunt	*Avr*II-P6.9-GUS-Pgp64-*Se*F-*Rsr*II-I-*Sce*I-*Cfr*9I	DH5α
KY9	pJET1/blunt	*Avr*II-P6.9-GUS-Pgp64-GP64-*Rsr*II-I-*Sce*I-*Cfr*9I	DH5α
KY13	KY3	*Avr*II-P6.9-GUS-*Rsr*II-I-*Sce*I-*Cfr*9I	DH10B
KY14	KY3	*Avr*II-P6.9-GUS-Pgp64-*Se*F-*Rsr*II-I-*Sce*I-*Cfr*9I	DH10B
KY15	KY3	*Avr*II-P6.9-GUS-Pgp64-GP64-*Rsr*II-I-*Sce*I-*Cfr*9I	DH10B
KY16	KY5	*Avr*II-P6.9-GUS-*Rsr*II-I-*Sce*I-*Cfr*9I	DH10B
KY17	KY5	*Avr*II-P6.9-GUS-Pgp64-*Se*F-*Rsr*II-I-*Sce*I-*Cfr*9I	DH10B
KY18	KY5	*Avr*II-P6.9-GUS-Pgp64-GP64-*Rsr*II-I-*Sce*I-*Cfr*9I	DH10B

Table 2.3 Animal cell lines and culturing conditions.

Cell line	**Source**	**Media**	**Supplements**	**Temp.**
Sf9	Insect	TMN-FH	10% CFBS*	27°C
HepG2	Human	MEM**	1% sodium pyruvate, 10% DFBS***	37°C
BHK-21	Murine	MEM	1% sodium pyruvate, 10% DFBS	37°C
PK-15	Porcine	MEM	1% sodium pyruvate, 10% DFBS	37°C
CHSE-214	Fish	MEM	10% CFBS	RT
EPC	Fish	MEM	10% CFBS	RT
McCOY	Mouse	Eagle's MEM	10% DFBS	37°C
FKC	Feline	Eagle's MEM	10% DFBS	37°C
SSN-1	Fish	Eagle's MEM	10% DFBS	RT
RTG-2	Fish	Eagle's MEM	10% DFBS	RT
ST	Porcine	Eagle's MEM	1% non-essential amino acid, 10% DFBS	37°C
VERO	Monkey	Eagle's Basal Medium	5% DFBS	37°C
MDCK	Canine	Alpha MEM	10% FDBS	37°C
MDBK	Bovine	MEM	5% Horse Serum	37°C
SCP	Sheep	MEM	5% Horse Serum	37°C
UMNSAH/DF-1	Chicken	DMEM	10% DFBS	39°C

*CFBS: characterized fetal bovine serum
**MEM: Minimum essential medium
***DFBS: Defined fetal bovine serum

2.3 Results

2.3.1 Generation of shuttle vectors carrying reporter and envelope protein genes.

A total of 13 plasmids (KY1, 3, 4, 5, 7-9, 13-18) carrying different combinations of mammalian reporter genes and baculovirus envelope protein genes were generated in this study (Table 2.2, Fig. 2.1). Six shuttle vectors (KY13~18) were used to insert the reporter genes and envelope protein genes into the cloned *Ac*MNPV genome (bacmid) by Tn*7*-mediated transposition. All six shuttle vectors were used to insert genes into a *gp64null-Ac*MNPV bacmid (*gp64null*-pMON14272, Lung *et al.,* 2002) in which the gene encoding the major BV envelope protein was deleted. Four shuttle vectors (KY13, 14, 16, and 17) were used to insert genes into the *Ac*MNPV bacmid (pMON14272) in which the *gp64* gene was intact. A schematic diagram of the bacmid constructs generated is illustrated in Fig 2.2. A total of ten recombinant bacmids, five with hrGFP reporter gene, and five with a Luc reporter gene were generated (Fig 2.2): b*gp64nullAc*Bac/ΔFBgus-CMV-hrGFP, and b*gp64nullAc*Bac/ΔFBgus-CMV-Luc (no envelope protein gene transposition); b*gp64nullAc*Bac/ΔFBgus-*Se*F-CMV-hrGFP and b*gp64nullAc*Bac/ΔFBgus-*Se*F-CMV-Luc (with *Se*F gene transposition); b*gp64nullAc*Bac/ΔFBgus-*GP64*-CMV-hrGFP and b*gp64nullAc*Bac/ΔFBgus-*GP64*-CMV-Luc (with *gp64* gene transposition); b*Ac*Bac/ΔFBgus-CMV-hrGFP and b*Ac*Bac/ΔFBgus-CMV-Luc (with endogenous *gp64* gene); b*Ac*Bac/ΔFBgus-*Se*F-CMV-hrGFP and b*Ac*Bac/ΔFBgus-*Se*F-CMV-Luc (with both the endogenous *gp64* gene and *Se*F gene transposition). The bacmids were confirmed by PCR using primer pairs specific for the reporter genes and the envelope protein genes (Fig. 2.3). The five recombinant bacmids with the hrGFP reporter gene were transfected into Sf9 cells to generate recombinant viruses that either had the endogenous envelope

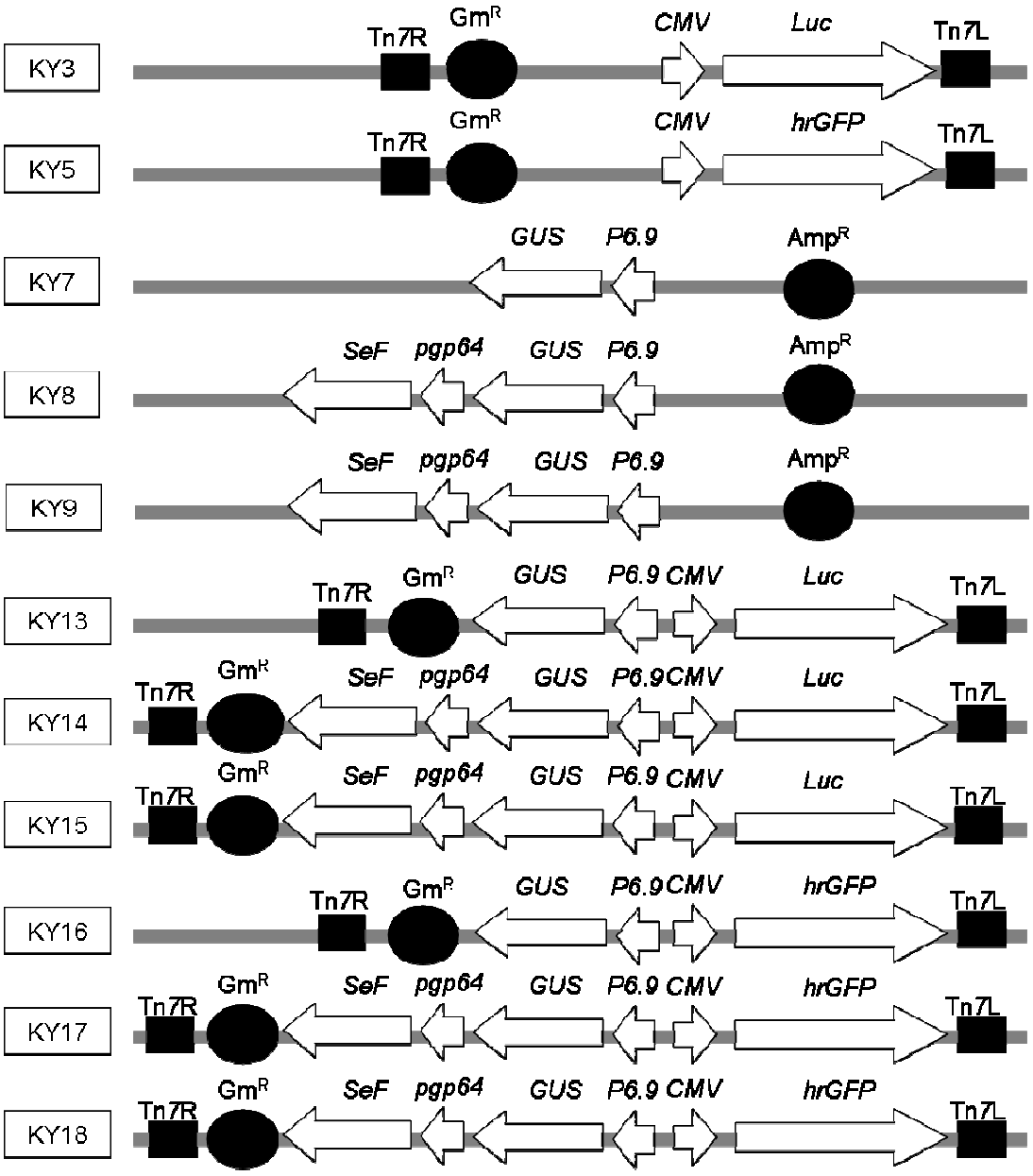

Fig 2.1 Linear maps of shuttle vectors carrying reporter genes and/or envelope genes generated for this study. KY3, 5, 13-18 were constructed from a shuttle vector, pFBDM, where the gene of interest was inserted in between the Tn7-transposition sites. KY7-9 were constructed from a PCR cloning vector, pJET1/blunt, where the PCR products can be directly cloned into the multiple cloning site. Gm^R= Gentamycin resistance gene, Amp^R= Ampicilline resistance gene, Tn7R (L) = Tn7-attachment right (left) site.

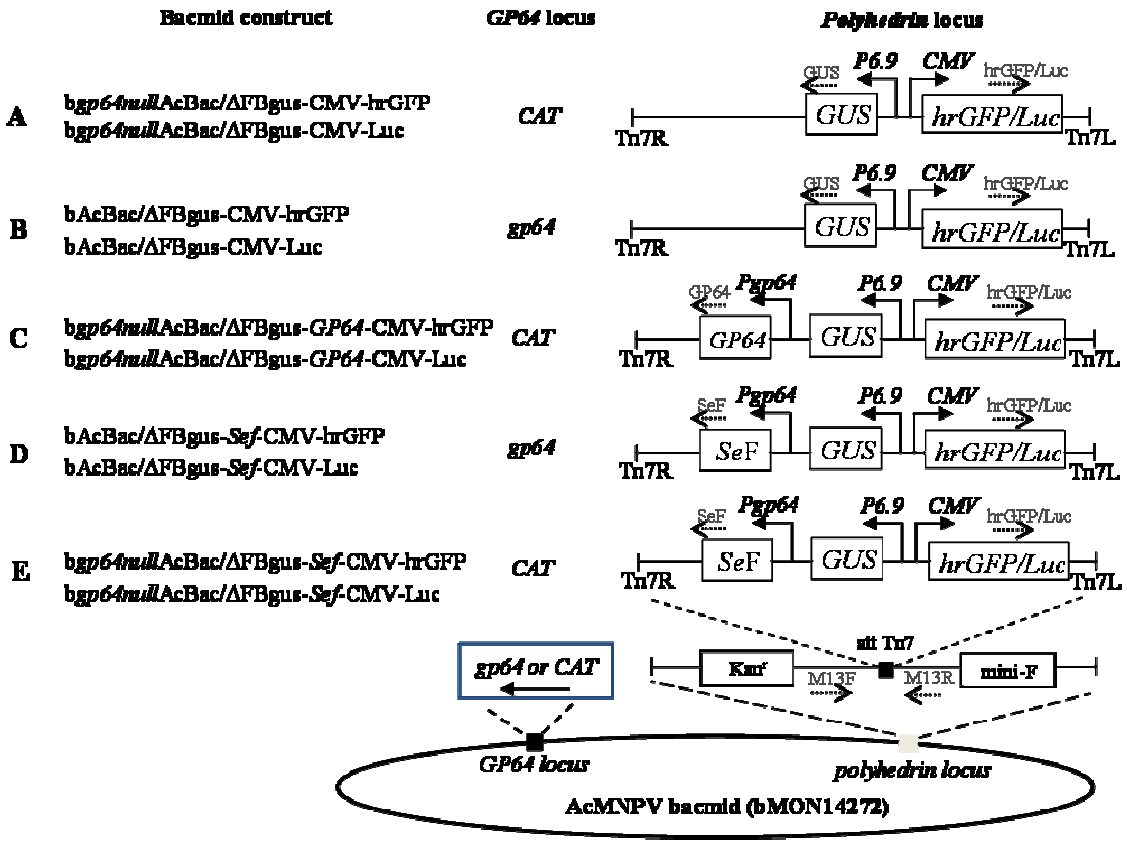

Fig 2.2 Schematic diagram of the ten bacmid constructs generated in this study. The *gp64* gene was replaced with the *CAT* gene in *gp64*null-*Ac*bacmid (A, C, D), while the *gp64* gene is intact in *Ac*bacmid (B, C). Insertion of hrGFP mammalian reporter genes (or alternatively, the Luc reporter gene) and GUS insect reporter genes into the *polyhedrin* locus was performed by Tn*7*-mediated transposition for all bacmid constructs. A *gp64* envelope protein gene (C) and a *Se*F envelope protein gene (D, E), both under the control of Pgp64 was inserted downstream of the GUS gene. PCR analysis were performed to ensure the transposition had taken place and also to rule out the presence of bacmids without any inserts. The relative position of the PCR primers used for bacmid confirmation is indicated (dashed arrows).

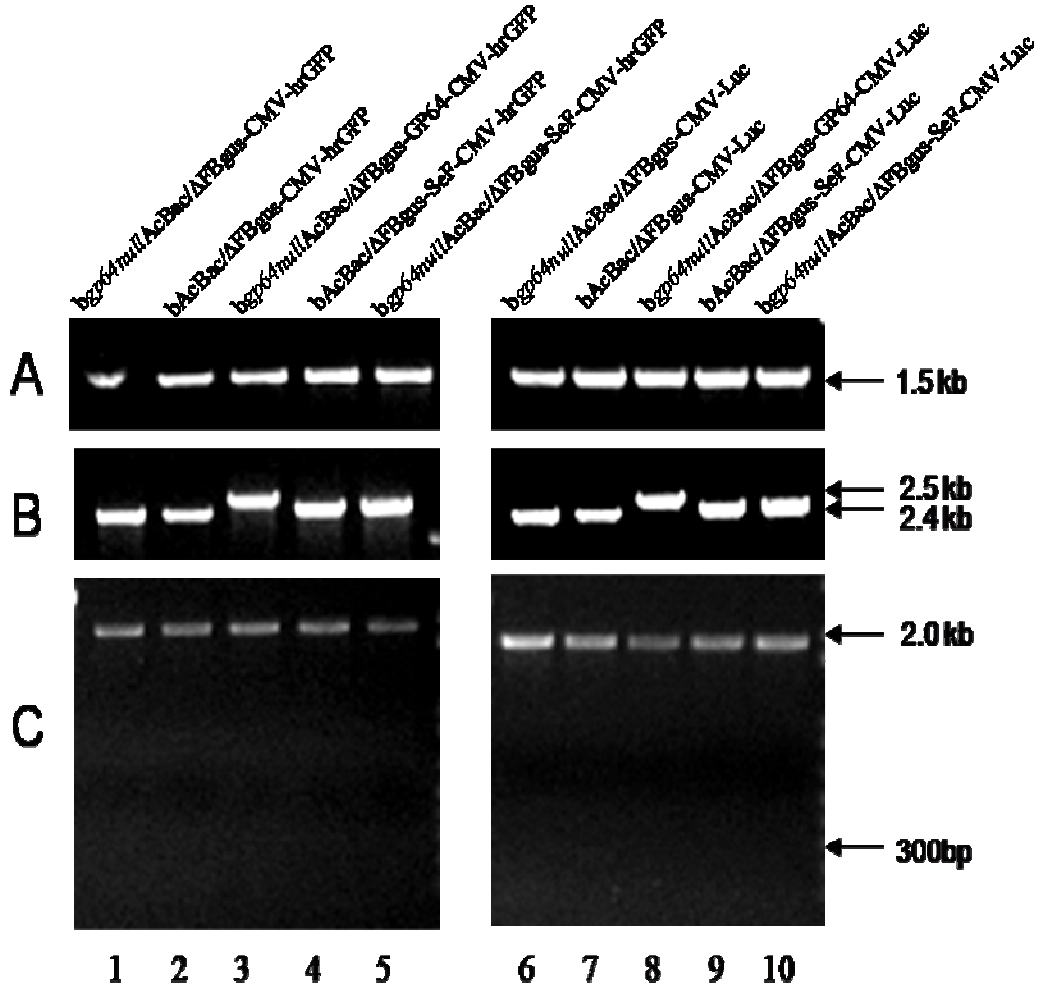

Fig 2.3 Confirmation of bacmid DNA by PCR analysis using: A) a M13 reverse primer and a hrGFP forward primer (lanes 1-5), or a M13 reverse primer and a Luciferase forward primer (lanes 6-10); B) a M13 forward primer and a GUS forward primer (lanes 1, 2, 6, 7), a M13 forward primer and a *gp64* forward primer (lanes 3, 8), a M13 forward primer and a *Se*F forward primer (lanes 4, 5, 9, 10); and C) a M13 forward and a M13 reverse primer. All PCR reactions gave the expected amplicon size and none of the bacmid-preparations produced a 300 bp amplicon with the M13 forward and M13 reverse primer, indicating there is no contamination with bacmid that did not undergo Tn*7* transposition.

protein GP64, the *Se*F protein instead of GP64, or had both GP64 and *Se*F. The recombinant viruses were confirmed by PCR analysis using primers specific to the GUS gene and the envelope protein genes (Fig. 2.4). A list of the recombinant viruses generated for this study is shown in Table 2.4. The recombinant viruses were also confirmed by Western blot analysis using antibodies against the envelope proteins GP64. As expected, Western blots of lysates of v*Ac*$^{\text{CMV-hrGFP}}$, v*gp64nullAc*$^{\text{GP64-CMV-hrGFP}}$, and v*Ac*$^{\textit{Se}\text{F-CMV-hrGFP}}$ infected cells probed with anti-GP64 had the predicted 64 kDa band, but the band was absent in lanes containing lysates from v*gp64nullAc*$^{\textit{Se}\text{F-CMV-hrGFP}}$ infected cells (Fig. 2.5). Since *Ac*MNPV will not propagate without GP64 or a functional substitute such as *Se*F (Lung *et al.,* 2002), the absence of GP64 in v*gp64nullAc*$^{\textit{Se}\text{F-CMV-hrGFP}}$ infected cell lysates strongly suggest that *Se*F protein is expressed.

2.3.2 GP64-containing *Ac*MNPV can transduce a variety of non-insect cell lines.

The transduction efficiencies of the four recombinant *Ac*MNPV variants were compared by counting the percentage of GFP positive cells 24 hour after transduction. Although the transduction efficiency is cell line dependent, results for a particular cell line show that the two GP64-containing and *Se*F negative viruses had comparable transduction efficiencies (Table 2.5, Fig. 2.6). GP64-containing virus transduced human HepG2 cells very efficiently, with greater than 96% of the cells positive for GFP expression (Fig. 2.7). The transduction efficiency of v*Ac*$^{\text{CMV-hrGFP}}$ was approximately 73.24% for monkey VERO cells, 63.74% for feline FKC cells, 55.33% for porcine PK-15 cells, 33.85% for porcine ST cells, 6.6% for canine MDCK cells, and 5.31% for chicken UMNSAH/DF-1 cells. Of the four fish-derived cell lines tested, the transduction efficiency was 2.2% for

RTG-2 cells, 13.29% for SSN-1 cells, 14.99% for CHES-214 cells, and 50.74% for EPC cells.

2.3.3 *Se*F pseudotyped *gp64null-Ac*MNPV do not transduce any of the non-insect cell lines efficiently.

Transduction of *Se*F-pseudotyped *gp64null-Ac*MNPV (v*gp64nullAc*$^{Se\text{F-CMV-hrGFP}}$) was done in parallel with the three *gp64*-containing viruses to examine whether *Se*F pseudotyping could be used to narrow the tropism of *Ac*MNPV for vertebrate cells. Results show the transduction efficiencies of the *Se*F-pseudotyped *gp64null* virus on all tested cell lines were lower than the control viruses (v*gp64nullAc*$^{\text{GP64-CMV-hrGFP}}$). At a moi of 500, the transduction efficiencies of v*gp64nullAc*$^{Se\text{F-CMV-hrGFP}}$ were highest on fish EPC, SSN-1 and CHES-214 cells, with efficiencies of approximately 1.31%, 5.22%, and 2.95%, respectively. The value for EPC is significantly lower than the 43.28% seen with the v*gp64nullAc*$^{\text{GP64-CMV-hrGFP}}$control virus. Expression of GFP was observed in less than 0.2% of the porcine ST, murine McCOY, feline FKC, monkey Vero, hamster BHK-21, and human HepG2 cells transduced with the v*gp64nullAc*$^{Se\text{F-CMV-hrGFP}}$ virus. These values represent a significant drop from the approximately 95% for HepG2 cells, 85% for BHK-21 cells, 69.32% for VERO cells, and 59.08% for FKC cells. No GFP positive cells were observed in canine MDCK, porcine PK-15, fish RTG-1, and chicken UNMSAH-DF-1 cells transduced with v*gp64nullAc*$^{Se\text{F-CMV-hrGFP}}$ whereas the transduction efficiency of PK-15 by the v*gp64nullAc*$^{\text{GP64-CMV-hrGFP}}$ control virus was 50.28% (Table. 2.5, Fig. 2.6). The low or lack of transduction by the v*gp64nullAc*$^{Se\text{F-CMV-hrGFP}}$ virus is not due to lack of functionality of the CMV-hrGFP cassette in these cells as cells transduced with the GP64-

containing variants showed GFP expression. To rule out the possibility that the lack of transduction is due to virus inactivation, post-transduction inoculums were added to insect Sf9 cells to assay for the presence of infectious virus. Results show that all post-transduction inoculums contain infectious viruses that are capable of infecting Sf9 cells, indicating the low transduction efficiencies observed with v*gp64nullAc*$^{Se\text{F-CMV-hrGFP}}$ were not due to virus inactivation (Fig 2.8).

Since several of the mammalian cell lines can be transduced inefficiently by the v*gp64nullAc*$^{Se\text{F-CMV-hrGFP}}$ virus at a moi of 500, transduction was also performed at a moi of 50 on HepG2, BHK-21, VERO, ST, FKC and McCOY cells to determine if a lower moi could eliminate transduction. Results show that no GFP positive cell was observed in six cell lines tested when transduced with v*gp64nullAc*$^{Se\text{F-CMV-hrGFP}}$at a moi of 50 (data not shown). These results indicate that the tropism of v*gp64nullAc*$^{Se\text{F-CMV-hrGFP}}$ for mammalian cells can be lowered further by using a lower virus to cell ratio.

2.3.4 Observation of Cell-cell fusion in bovine MDBK and ovine SCP cells transduced with GP64-containing *Ac*MNPV.

An interesting observation made during this study was that bovine MDBK and sheep SCP cells transduced with GP64-containing viruses fused into large syncytiums by 24 hour post transduction (hpt). Cell fusion was not observed in experiments where cells were transduced with v*gp64nullAc*$^{Se\text{F-CMV-hrGFP}}$, which does not have GP64, indicating the fusion of MDBK and SCP cells is due to the presence of GP64. When the kinetics of syncytium formation was examined by observing cells at various time points after a two

hour transduction by vAc$^{CMV\text{-}hrGFP}$, cell-cell fusion of MDBK cells was first observed at 3hpt (Fig. 2.9). These results suggest that under certain circumstances, GP64 may have membrane fusion activity in the absence of low pH activation.

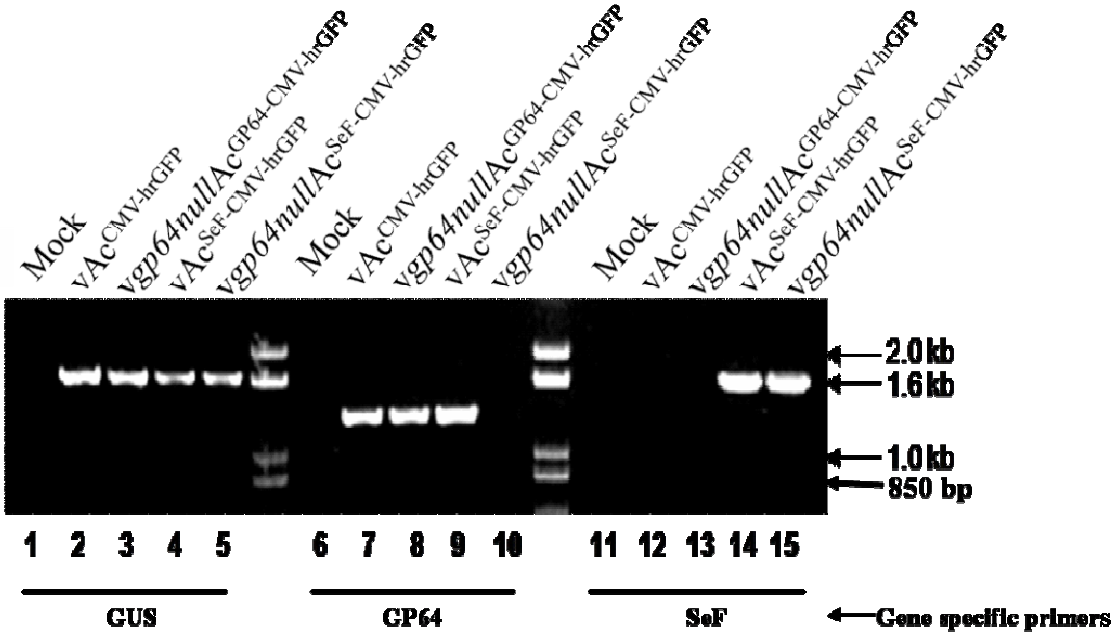

Fig. 2.4 Confirmation of recombinant *Ac*MNPV variants by PCR analysis. Viral DNA were extracted from Sf9 cells that were either mock infected (lanes 1, 6, 11), or infected with v*Ac*$^{\text{CMV-hrGFP}}$ (lanes 2, 7, 12), v*gp64nullAc*$^{\text{GP64-CMV-hrGFP}}$ (lanes 3, 8, 13), v*Ac*$^{\textit{Se}\text{F-CMV-hrGFP}}$ (lanes 4, 9, 14), v*gp64nullAc*$^{\textit{Se}\text{F-CMV-hrGFP}}$ virus (lanes 5, 10, 15), and amplified by PCR using primer pairs specific to the GUS gene (lanes 1-5), the *gp64* gene (lanes 6-10), or the *Se*F gene (lanes 11-15).

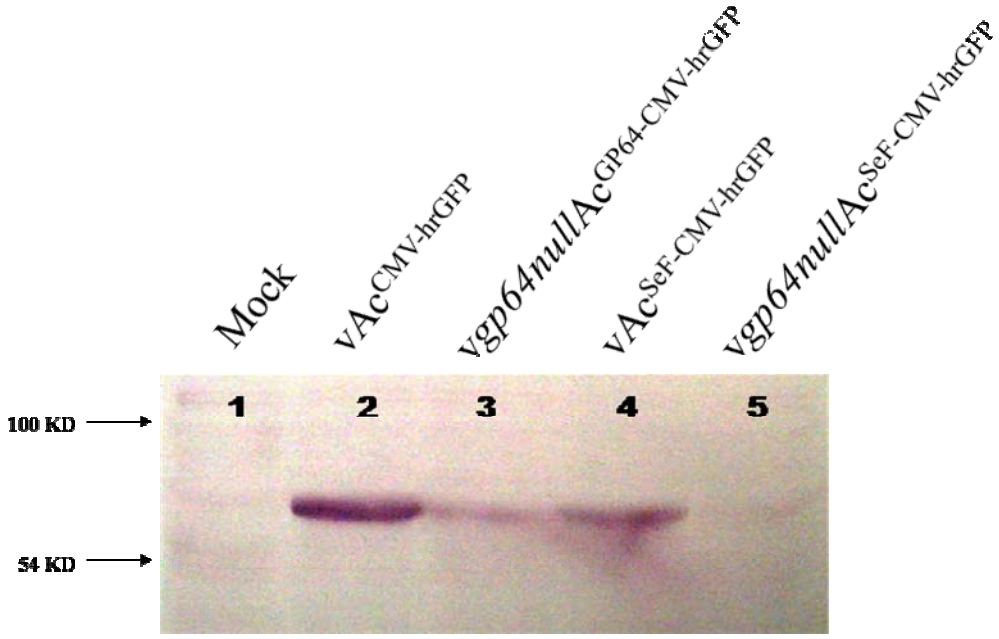

Fig. 2.5 Western-blot analysis of cell lysates from mock infected Sf9 cells (lane 1), Sf9 cells infected with v*Ac*$^{\text{CMV-hrGFP}}$ (lane 2), v*gp64nullAc*$^{\text{GP64-CMV-hrGFP}}$ (lane 3), v*Ac*$^{\textit{Se}\text{F-CMV-hrGFP}}$ (lane 4), and v*gp64nullAc*$^{\textit{Se}\text{F-CMV-hrGFP}}$ (lane 5). Cells were infected at a moi of one and cell lysates were harvested at 72 hpi. The Western blot was probed with an anti-GP64 monoclonal antibody (*Ac*V5).

Table 2.4 Virus generated in this study**.**

Virus	**mammalian reporter gene**	**envelope protein genes (locus)**	**Bacmid backbone**
v*gp64nullAc*$^{\text{CMV-hrGFP}}$	hrGFP	none	*gp64null*-bMON14272
v*Ac*$^{\text{CMV-hrGFP}}$	hrGFP	*gp64* (*gp64* locus)	DH10-bMON14272
v*gp64nullAc*$^{\text{GP64-CMV-hrGFP}}$	hrGFP	*gp64* (*polyhedrin* locus)	*gp64null*-bMON14272
v*Ac*$^{\textit{Se}\text{F-CMV-hrGFP}}$	hrGFP	*gp64* (*gp64* locus) + *Se*F (*polyhedrin* locus)	DH10-bMON14272
v*gp64nullAc*$^{\textit{Se}\text{F-CMV-hrGFP}}$	hrGFP	*Se*F (*polyhedrin* locus)	*gp64null*-bMON14272

Table 2.5 Transduction efficiencies of four recombinant *Ac*MNPV variants on 14 different cell lines (500 moi).

Source/Cell	Tissue of origin	Virus	Cell count	Transduction efficiency (%)	SD
Human		v*Ac*$^{\text{CMV-hrGFP}}$	~358	96.9%	ND*
HepG2	Liver	v*gp64nullAc*$^{\text{GP64-CMV-hrGFP}}$	~718	98.47%	ND*
		v*Ac*$^{\textit{Se}\text{F-CMV-hrGFP}}$	~622	99.68%	ND*
		v*gp64nullAc*$^{\textit{Se}\text{F-CMV-hrGFP}}$	~1642	0.15%	ND*
Monkey		v*Ac*$^{\text{CMV-hrGFP}}$	1468	73.24%	9.08%
VERO	Kidney	v*gp64nullAc*$^{\text{GP64-CMV-hrGFP}}$	1558	69.32%	12.48%
		v*Ac*$^{\textit{Se}\text{F-CMV-hrGFP}}$	1852	84.02%	6.07%
		v*gp64nullAc*$^{\textit{Se}\text{F-CMV-hrGFP}}$	2202	0.18%	0.31%
Mouse		v*Ac*$^{\text{CMV-hrGFP}}$	2440	2.91%	1.2%
McCOY	Fibroblasts	v*gp64nullAc*$^{\text{GP64-CMV-hrGFP}}$	1945	5.91%	4.86%
		v*Ac*$^{\textit{Se}\text{F-CMV-hrGFP}}$	2487	7.64%	1.6%
		v*gp64nullAc*$^{\textit{Se}\text{F-CMV-hrGFP}}$	2290	0.13%	0.28%
Porcine		v*Ac*$^{\text{CMV-hrGFP}}$	600	55.33%	14.64%
PK-15	Kidney	v*gp64nullAc*$^{\text{GP64-CMV-hrGFP}}$	535	50.28%	13.96%
		v*Ac*$^{\textit{Se}\text{F-CMV-hrGFP}}$	609	55.01%	8.27%
		v*gp64nullAc*$^{\textit{Se}\text{F-CMV-hrGFP}}$	539	0.00%	0.00%
ST	Testicle	v*Ac*$^{\text{CMV-hrGFP}}$	1681	33.85%	7.01%
		v*gp64nullAc*$^{\text{GP64-CMV-hrGFP}}$	1658	41.25%	8.21%
		v*Ac*$^{\textit{Se}\text{F-CMV-hrGFP}}$	1124	39.5%	6.78%
		v*gp64nullAc*$^{\textit{Se}\text{F-CMV-hrGFP}}$	1469	0.14%	0.31%
Feline		v*Ac*$^{\text{CMV-hrGFP}}$	1536	63.74%	5.60%
FKC	Kidney	v*gp64nullAc*$^{\text{GP64-CMV-hrGFP}}$	1200	59.08%	8.12%
		v*Ac*$^{\textit{Se}\text{F-CMV-hrGFP}}$	1162	64.20%	5.68%
		v*gp64nullAc*$^{\textit{Se}\text{F-CMV-hrGFP}}$	1180	0.17%	0.42%
Canine		v*Ac*$^{\text{CMV-hrGFP}}$	788	6.60%	6.56%
MDCK	Kidney epithelial Kidney	v*gp64nullAc*$^{\text{GP64-CMV-hrGFP}}$	986	6.39%	4.51%
		v*Ac*$^{\textit{Se}\text{F-CMV-hrGFP}}$	935	5.35%	4.49%
		v*gp64nullAc*$^{\textit{Se}\text{F-CMV-hrGFP}}$	884	0.00%	0.00%
Chicken		*vAc*$^{\text{CMV-hrGFP}}$	1093	5.31%	3.38%
UMNSAH/DF-1	Embryo Fibroblast	v*gp64nullAc*$^{\text{GP64-CMV-hrGFP}}$	898	10.13%	2.39%
		v*Ac*$^{\textit{Se}\text{F-CMV-hrGFP}}$	1066	7.41%	4.62%
		v*gp64nullAc*$^{\textit{Se}\text{F-CMV-hrGFP}}$	451	0.00%	0.00%

Bovine		v*Ac*$^{\text{CMV-hrGFP}}$			
		v*gp64nullAc*$^{\text{GP64-CMV-hrGFP}}$		Cell fused	
MDBK	Kidney	v*Ac*$^{\textit{Se}\text{F-CMV-hrGFP}}$			
		v*gp64nullAc*$^{\textit{Se}\text{F-CMV-hrGFP}}$	1456	0.34%	0.57%
Ovine		v*Ac*$^{\text{CMV-hrGFP}}$			
		v*gp64nullAc*$^{\text{GP64-CMV-hrGFP}}$		Cell fused	
SCP	Choroid plexus	v*Ac*$^{\textit{Se}\text{F-CMV-hrGFP}}$			
		v*gp64nullAc*$^{\textit{Se}\text{F-CMV-hrGFP}}$	~2042	0%	0%
Fish		v*Ac*$^{\text{CMV-hrGFP}}$	1089	2.20%	1.76%
		v*gp64nullAc*$^{\text{GP64-CMV-hrGFP}}$	1155	3.03%	3.91%
RTG-2	Gonad	v*Ac*$^{\textit{Se}\text{F-CMV-hrGFP}}$	1176	1.87%	1.05%
		v*gp64nullAc*$^{\textit{Se}\text{F-CMV-hrGFP}}$	950	0.00%	0.00%
		v*Ac*$^{\text{CMV-hrGFP}}$	1293	13.29%	3.85%
	Whole fry tissue	v*gp64nullAc*$^{\text{GP64-CMV-hrGFP}}$	1744	7.65%	2.6%
SSN-1		v*Ac*$^{\textit{Se}\text{F-CMV-hrGFP}}$	1294	17.21%	8.04%
		v*gp64nullAc*$^{\textit{Se}\text{F-CMV-hrGFP}}$	1519	5.22%	1.68%
		v*Ac*$^{\text{CMV-hrGFP}}$	1488	14.99%	15.34%
CHES-214	Salmon embryos	v*gp64nullAc*$^{\text{GP64-CMV-hrGFP}}$	1432	19.41%	3.55%
		v*Ac*$^{\textit{Se}\text{F-CMV-hrGFP}}$	1289	26.76%	6.16%
		v*gp64nullAc*$^{\textit{Se}\text{F-CMV-hrGFP}}$	1864	2.95%	1.63%
		v*Ac*$^{\text{CMV-hrGFP}}$	339	50.74%	10.58%
EPC	Epithelioma	v*gp64nullAc*$^{\text{GP64-CMV-hrGFP}}$	67	43.28%	15.89%
		v*Ac*$^{\textit{Se}\text{F-CMV-hrGFP}}$	129	46.51%	8.46%
		v*gp64nullAc*$^{\textit{Se}\text{F-CMV-hrGFP}}$	689	1.31%	1.54%

***ND:** not determinated**.**

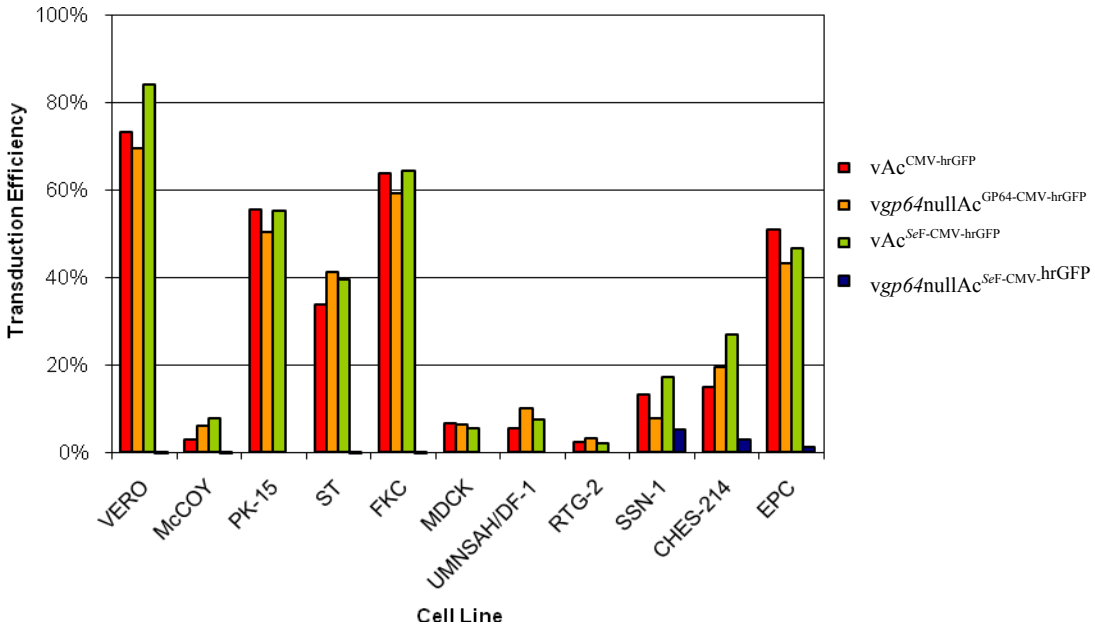

Fig 2.6 Transduction efficiencies of 11 vertebrate cell lines by four recombinant AcMNPV variants. Cells were scored for GFP expression at 24 hpt transduction with vAc$^{\text{CMV-hrGFP}}$ (red), v*gp64nullAc*$^{\text{GP64-CMV-hrGFP}}$ (orange), v*Ac*$^{\textit{Se}\text{F-CMV-hrGFP}}$ (green), and v*gp64nullAc*$^{\textit{Se}\text{F-CMV-hrGFP}}$. Transduction efficiencies were not shown for HepG2, MDBK, and SCP due to the cell clumping of HepG2 cells and cell-cell fushion for MDBK cells and SCP cells.

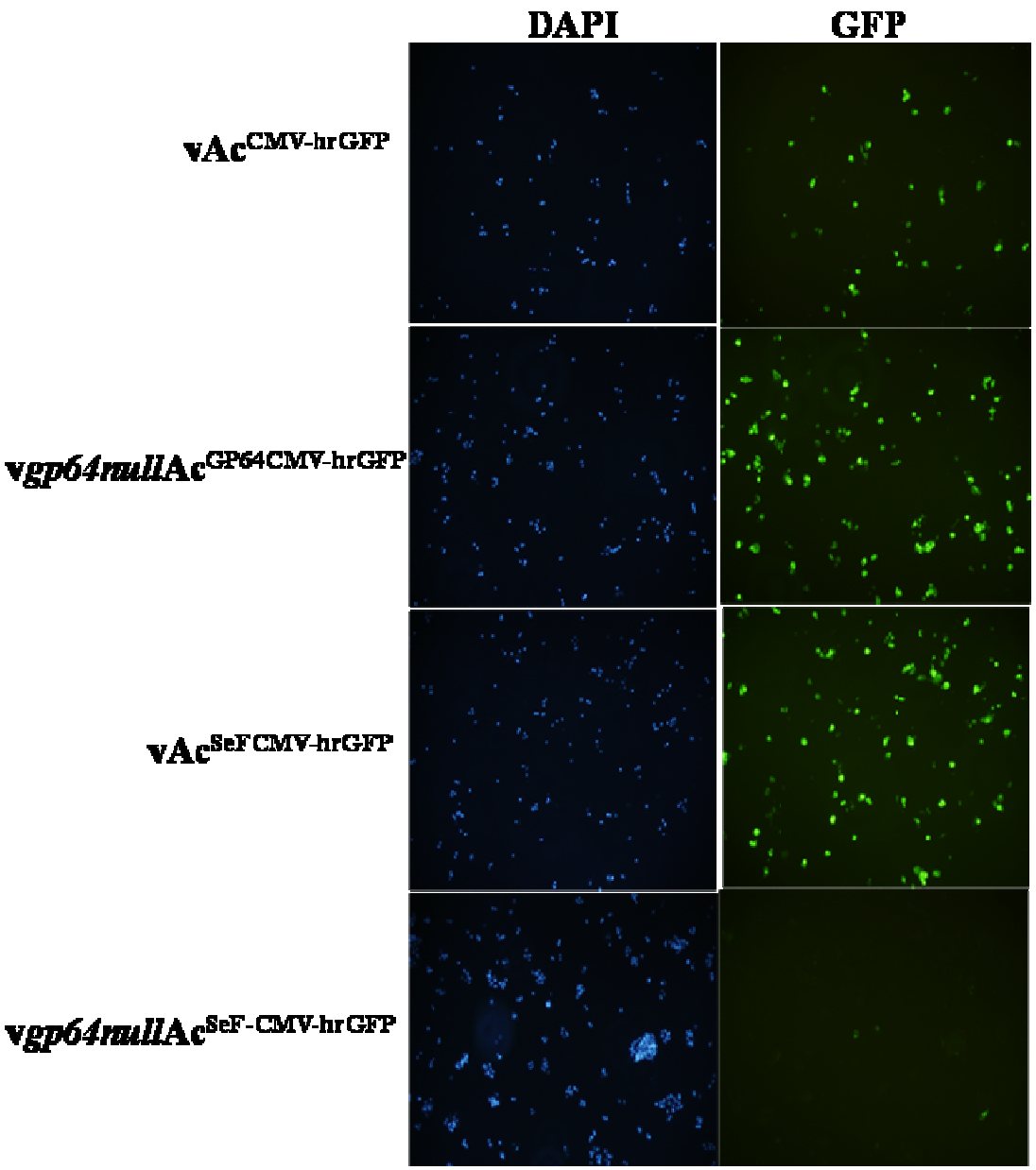

Fig. 2.7 Representative fluorescent micrographs of human HepG2 cells transduced with the four *Ac*MNPV variants. DAPI fluorescence of stained cell nuclei is shown in the left column, and GFP fluorescence in shown in the right column. The viral variant used for the transduction is shown on the left of the images.

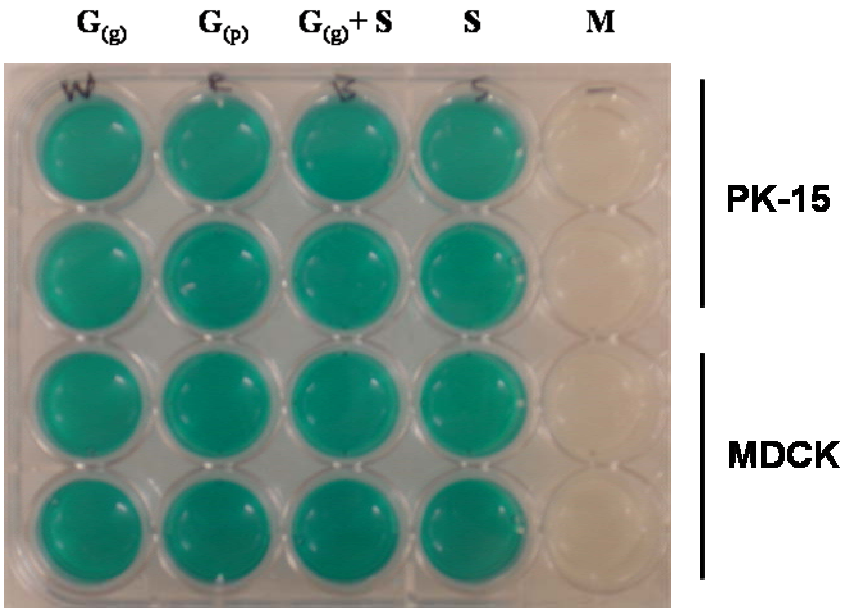

Fig. 2.8 Representative example of GUS activity assay performed to confirm the presence of infectious virions in the post-transduction inoculums. Post-transduction inoculums from PK-15 and MDCK cells that were mock transduced (M), or transduced with v*Ac*$^{\text{CMV-hrGFP}}$ ($G_{(g)}$), v*gp64null Ac*$^{\text{GP64-CMV-hrGFP}}$ ($G_{(p)}$), v*Ac*$^{\textit{Se}\text{F-CMV-hrGFP}}$, ($G_{(g)}$+S), v*gp64*null*Ac*$^{\textit{Se}\text{F-CMV-hrGFP}}$ (S) were added to Sf9 cells. Substrate for GUS was added to the Sf9 cells at 3 day post infection to detect GUS expression in virus infected cells.

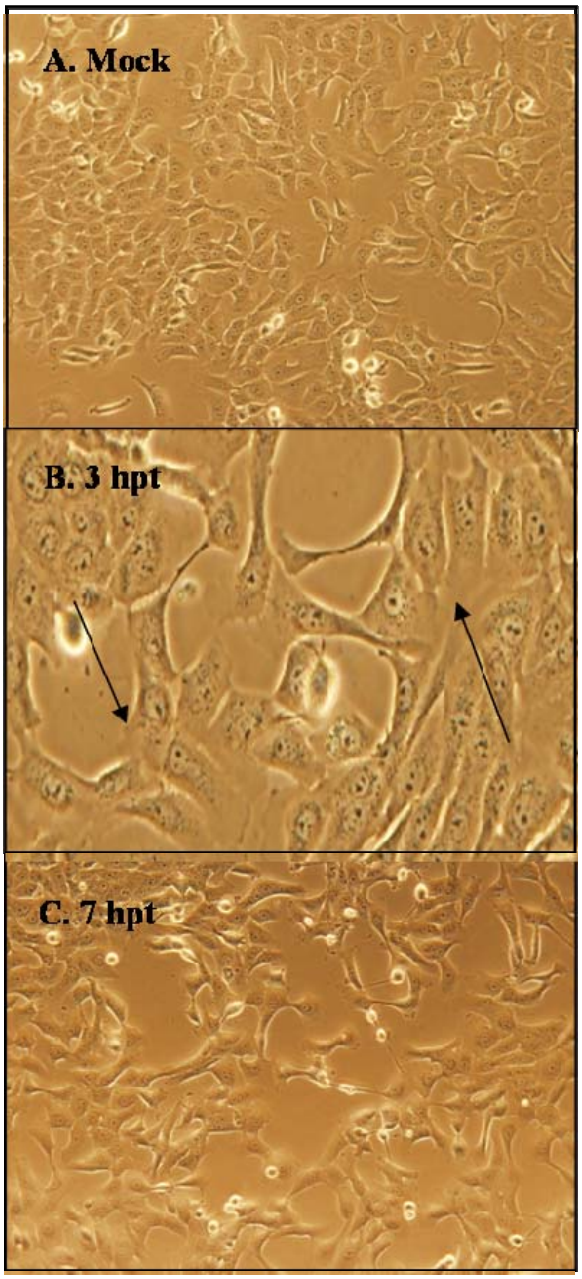

Fig. 2.9 Syncytium formation in MDBK cells after transduction with $vAc^{CMV\text{-}hrGFP}$. A) mock transduced, B) 3 C) 7 hour post transduction (hpt) with $vAc^{CMV\text{-}hrGFP}$. Cell-cell fusion can be clearly observed at 3 hpt (arrow).

2.4 Discussion

Several lines of evidence suggest that GP64, the envelope protein of *Ac*MNPV BV play a critical role in the transduction of non-insect cells by *Ac*MNPV (Tani *et al*., 2001; Liang *et al*., 2005). However, similar evidence suggests that F proteins from group II NPV can not mediate viral entry into mammalian cells (Liang *et al.,* 2005; Westenberg *et al.,* 2007). To examine the impact GP64 and F have on transduction of vetebrate cells, recombinant *Ac*MNPV viruses with either a single copy of the *gp64* gene, a single copy of the *Se*F gene, or with a single copy of both genes were generated. The transduction efficiency of four recombinant *Ac*MNPV variants with a different repertoire of envelope proteins were tested on 15 cell lines derived from human, monkey, porcine, feline, canine, bovine, ovine, avian, and fish species. Transduction of *Se*F-pseudotyped *gp64null-Ac*MNPV is reported for the first time for 12 cell lines and the transduction efficiency for *Ac*MNPV with the endogenous GP64 protein is reported for the first time for feline FKC (59.8%), porcine ST (33.85%), chicken UMNSAH/DF-1 (5.31%), and fish SSN-1 (13.29%) cells in this study. Furthermore, transduction of vertebrate cells by AcMNPV carrying both GP64 and *Se*F envelope proteins are also reported for the first time. Results show that GP64-containing *Ac*MNPV can efficienctly transduce liver (HepG2) and many kidney-derived cell lines (BHK-21, VERO, PK-15, FKC) except for the two kidney epithelial cell lines (canine MDCK and bovine MDBK). These results are consistent with a previous report that transduction efficiency of hepatocyte-derived cell lines (Huh7, HepG2) and primary hepatocytes (phH, pw-rH) using recombinant AcMNPV carrying a CMV-luciferase reporter gene were significantly higher than other

cell lines tested, whereas transduction of MDCK cells is very poor (Hofmann *et al.,* 1995). A previous report has also shown that kidney-derived cell lines (BHK, 293, COS-7, PK-15) and primary mouse kidney cells (MKC) could be efficiently transduced with *Ac*-CMV-GFP (Liang *et al.,* 2004). The results presented here also showed that all three of the *gp64*-containing viruses can transduce fish EPC (43%~51%) and CHES-214 (17%~27%) cells with moderate efficiency and RTG-2 cells with low efficiency (1.87%~3.3%). This is somewhat unexpected since it has been reported that transduction efficiency of fish EPC (0.04-2%) and CHES-214 (0.05%) cells was extremely low, and undetectable for RTG-2 cells when transduced with a fairly similar virus to ones used in this study (Ac-CA*lacZ*), but carrying a different reporter gene cassette (Leisy *et al.,* 2003). This inconsistency is most likely due to the different promoter used to drive reporter gene expression. A CMV promoter was used in this study, while the CAG promoter was used by Leisy et al. (2003). The higher moi (500 vs. 200), and the longer duration of transduction (6 hour vs. 1 hour) used in this study may also have contributed to the higher transduction efficiency observed in this study. Thus my results indicate fish cell lines are susceptible to *Ac*MNPV transduction.

*Se*F-pseudotyped *gp64null-Ac*MNPV transduction of cell lines derived from chicken, fish and mammalian species were done in parallel with three *gp64*-containing control viruses. All cell lines tested showed little or no transduction by the v*gp64nullAc*$^{Se\text{F-CMV-hrGFP}}$ virus. This virus did not appear to transduce chicken UMNSAH/DF-1, canine MDCK, porcine PK-15, and fish RTG-2 cells. Some transduction of porcine ST, mouse McCOY, feline FKC, monkey Vero, hamster BHK-21, and human HepG2 cells was detected but the

efficiency was low (< 0.2%). However, transduction of ST and McCOY cells were not detected when transduction was carried out using a lower moi of 50, indicating the transduction of these cell types can be further diminished by using a lower virus to cell ratio. The three fish cell lines (EPC, SSN-1 and CHES-214) had the highest transduction efficiency of the cell lines tested (1.31-5.22%). The inefficient or lack of transduction by the *Se*F-pseudotyped *gp64null-Ac*MNPV is not due to inactivation of the viruses, nor was it due to lack of CMV-hrGFP reporter cassette activity in these cell lines. These results indicate that *Se*F-pseudotyped *gp64null-Ac*MNPV have significantly narrower tropism for vetebrate cells than *Ac*MNPV with the endogenous envelope protein GP64. The presence of *Se*F in addition to GP64 on the virion did not significantly increase the transduction efficiency of most cell lines. However, transduction efficiencies of VERO, McCOY, ST, FKC, SSN-1 and CHES-214 cells were slightly increased when both envelope proteins are present (v*Ac*$^{Se\text{F-CMV-hrGFP}}$). Interestingly, these were the cell lines that showed detectable transduction by the viruse with *Se*F in place of GP64, suggesting that the presence of *Se*F can enhance the transduction of certain cell lines that are transduced by the *Se*F-pseudotyped *gp64null-Ac*MNPV virus. Consistent with this hypothesis, the presence of *Se*F did not effect the transduction efficiency of most other cell lines (PK-15, MDCK, UMNSAH/DF-1, RTG-2) that were not transduced by *Se*F-pseudotyped *gp64null-Ac*MNPV.

Recently Westenberg et al. (2007) reported that *Se*F-pseudotyped *gp64null-Ac*MNPV carrying a CMV-eGFP reporter cassette showed no detectable GFP fluorescence in mammalian BHK-21, LLC-PK1, and H35 cells when the transduction was performed at

37°C for 2 hours using a moi of 100. In this study, positive but extremely low transduction efficiency was found in mammalian BHK-21, as well as ST, McCOY, FKC, Vero, and HepG2 cells. This inconsistency might be due to the following reasons: 1) the higher transduction moi used in this study (500 moi vs. 100 moi); 2) a longer transduction duration used in this study (6 hour vs. 2 hour); and 3) differences in the temperature and the media in which transduction was carried out. The transduction media and temperature used in this study (25°C in Dulbecco's phosphate-buffered saline , D-PBS) has been reported to give higher transduction efficiency when compared with the tranditional transduction protocol (37°C in cell culture medium) used by Westenberg *et al.* (Hsu *et al.,* 2004).

GP64 has been well characterized to be a low-pH dependent membrane fusion protein in insect cells (Blissard and Wenz, 1992). Membrane fusion by GP64 in the absence of low pH activation has not been reported. My observation that MDBK and SCP cells can be induced to fuse under neutral pH after *Ac*MNPV transduction, suggest that GP64 may mediate cell fusion without low-pH activation under certain circumstances. The mechanism by which GP64 mediates fusion of these cells remains to be determined.

Kitagawa *et al*. (2005) showed that *gp64null-Ac*MNPV pseudotyped with CD46 or SLAM (receptors of measles virus) can specifically transduce BHK cells expressing measles virus envelope glycoproteins, indicating that pseudotyped *gp64*-null *Ac*MNPV displaying targeting molecules can be used for targeted gene delivery. However, these pseudotyped *gp64*-null *Ac*MNPVs lack the essential *gp64* gene, can not be amplified, and

therefore have very limited practical use. Since *Se*F-pseudotyped *gp64null-Ac*MNPV can be amplified to high titer, and appears to significantly narrow the tropism for mammalian cell lines, this virus may be useful as a platform for further addition of targeting molecules for targeted gene delivery. Additional advantages of the *Se*F-pseudotyped *gp64null-Ac*MNPV platform for target cell-specific gene delivery include the fact that targeting molecules (e.g., ligands or envelope protein of heterologous virus) can be easily engineered into the *Se*F-pseudotyped *gp64null-Ac*MNPV genome for ligand-directed cell targeting. Strategies such as the engineering of viral surface proteins can be further combined with the use of tissue-specific promoters for tissue specific gene expression to further decrease detrimental effects that may be caused by misexpression of transgenes in unintended target cells.

Chapter 3. *Autographa californica* Multicapsid Nucleopolyhedrovirus (*Ac*MNPV) ORF 23 null mutant produces occlusion-derived virions with fewer nucleocapsids.

3.1 Introduction.

Baculovirus constitute a large and diverse family of enveloped viruses with rod shaped nucleocapsids, and a circular double stranded DNA genome that ranges in size from 80 kbp to 180 kbp (Blissard *et al.,* 2000). Baculovirus are restricted to arthropod hosts and have been isolated from more than 600 host species. The type baculovirus, *Autographa californica* multicapsid nucleopolyhedrovirus (*Ac*MNPV), has a biphasic life cycle in which two virion phenotypes with distinctively different roles are produced in the same cell (Miller *et al.,* 1997). Budded viruses (BVs) are produced early in infection when nucleocapsids acquire an envelope by budding through the plasma membrane of the infected cell. BVs infect a variety of cell types and are responsible for the systemic spread of viral infection within a host (Granados *et al.,* 1981; Engelhard *et al.,* 1994; Federici *et al.,* 1997; Mangor *et al.,* 2001). In contrast, occlusion-derived virions (ODVs) are produced late in infection when nucleocapsids become enveloped within the nucleus (for review, see reference Slack and Arif, 2006). ODVs are subsequently encapsulated within the nucleus by polyhedrin proteins to form proteinaceous, polyhedral crystal-like structures called occlusion bodies (OBs). OBs are released upon host cell lysis and are believed to protect ODVs from detrimental factors present in the dying host and the environment. Upon ingestion of OBs by susceptible hosts, the alkaline environment of the host's midgut triggers OB disassembly and ODV release. ODVs are highly infectious

to midgut epithelial cells and establish the primary infection in the animal midgut after which BVs spread the infection systemically (Granados *et al.,* 1981; Keddie *et al.,* 1985, 1989; Mangor *et al.,* 2001; Monsma *et al.,* 1996).

Baculovirus are currently classified into two genera; the *Nucleopolyhedroviruses* (NPVs) (Rohrmann*,* 1999) and the *Granuloviruses* (GVs) (Wormleaton *et al.,* 2003). NPVs from Lepidopteran hosts have been further classified into group I and group II based on molecular phylogenetic analysis (Bulach *et al.,* 1999; Herniou *et al.,* 2001, 2003; Zanotto *et al.,* 1993). The NPVs are also further divided into single capsid nucleopolyhedroviruses (SNPVs) and multicapsid nucleopolyhedroviruses (MNPVs). SNPV ODVs contain a single nucleocapsid, while MNPV ODVs contain multiple nucleocapsids. The biological basis for SNPV and MNPV has not been determined, and it has been suggested that the single and multiple capsid morphotypes have no phylogenetic relevance (Herniou *et al.,* 2003). However, biological assays done on related, but not identical MNPV and SNPV virus, support the hypothesis that the MNPV has advantages over the SNPV in *per os* infectivity (Washburn *et al.,* 1999, 2003).

Envelope fusion proteins are important structural proteins that mediate entry of enveloped viruses into host cells. BVs enter by receptor-mediated endocytosis and their envelope proteins mediate fusion of the viral envelope with endosome membranes after endosomal acidification (Blissard and Wenz, 1992; Volkman and Goldsmith*.,* 1985). Two different BV envelope fusion proteins have been identified in the family baculoviridae: GP64 and F protein (for review, see reference Pearson and Rohrmann, 2002). GP64 has

been well characterized to be a low pH-activated membrane fusion protein that is essential for BV attachment, fusion and budding (Hefferon *et al.,* 1999; Oomens *et al.,* 1999). Early baculovirus literature based mostly on the group I baculovirus *Ac*MNPV and *Op*MNPV pointed to GP64 as the primary baculovirus BV envelope fusion protein. However it was later discovered that the genomes of group II NPVs such *Ld*MNPV and *Se*MNPV (IJkel *et al.,* 1999; Kuzio *et al.,* 1999) lacked *gp64* homologues. This led to the identification of *f* (short for fusion) genes. The *f* genes show no sequence similarity to *gp64*, but *Ld*MNPV F protein (Ld F or Ld130) and *Se*MNPV F protein (*Se*F or Se8) both had low pH-activated membrane fusion activity (IJkel *et al.,* 2000; Pearson *et al.,* 2000), and could rescue a *gp64*-null *Ac*MNPV mutant virus (Lung *et al.,* 2002). All sequenced group II NPVs, GVs and Dipteran baculovirus lack *gp64* homologues and have *f* gene homologs (Hiscock *et al.,* 2000). The only sequenced baculovirus genomes which lack both *f* genes and *gp64* genes are three Hymenopteran (sawfly) baculovirus (*Neab*NPV, *Nese*NPV and *Nele*NPV) (Duffy *et al.,* 2007; Garcia-Maruniak *et al.,* 2004; Keddie *et al.,* 1985). These viruses may however lack the BV phenotype.

Despite their having *gp64* gene homologues, *f* gene homologs are also found in group I NPV baculovirus genomes. Even more intriguing was the discovery that the F homologs of group I NPVs, *Ac*MNPV (Ac23) and *Op*MNPV (Op21) do not have detectable membrane fusion activity (Lung *et al*., 2003; Pearson *et al.,* 2000). Since the *f* genes are more divergent and more widely distributed within the baculoviridae than *gp64*, it has been proposed that *gp64* was a more recent acquisition by ancestors of group I NPVs (Pearson *et al.,* 2000) and that *gp64* subsequently supplanted the membrane fusion

function of the F protein. Homologs of the *f* gene have been identified in several Dipteran and Lepidopteran insect species suggesting that *f* genes may have been acquired from an insect host (Lung *et al.,* 2005). Characterization of an *Ac23*-null mutant *Ac*MNPV virus showed that this gene is not essential for viral replication and infectivity in cultured cells or in animals (Lung *et al.,* 2003). However, animals infected with *Ac23*-null mutant viruses survived longer (Lung *et al.,* 2003). In the present study we used confocal microscopy to show that *Ac23*-GFP localizes to the nuclear envelope, and electron microscopy (EM) to show that absence of *Ac23* correlated with an alteration in ODV composition (nucleocapsid number). How this in turn, affects pathogenesis in host animals will be discussed.

3.2 Materials and Methods

3.2.1. Cell culture and virus production.

Spodoptera frugiperda (Sf9) cells were cultured at 27°C in TNMFH supplemented with 10% (v/v) fetal bovine serum (Hyclone, Logan, UT). The *Ac*MNPV strain E2 virus was used as a wildtype (*wt*) control in the analysis along with three genetically modified bacmid-derived viruses. All viruses were amplified using Sf9 cells, and viral titers were determined by endpoint dilution. All three of the bacmid-derived viruses are derived from the bacmid bMON14272 (Invitrogen, Carlsbad, CA) and have been described previously (Lung *et al.,* 2003). The genome of the bacmid-derived control virus v*Ac*bacmid$^{/GUS+PH}$ (*Ac*bacmid) was generated by Tn*7*-mediated transposition of the vector pΔFBgus(R)-polyhedrin which contained a *polyhedrin* gene under the control of a

polyhedrin promoter and a *GUS* reporter gene under the control of a P6.9 promoter into the bacmid bMON14272. The v*Ac23*null$^{/GUS+PH}$ (*Ac23*null) mutant virus genome was generated by replacing the *Ac23* gene in bMON14272 with a *CAT* gene, and an insertion of the *polyhedrin* gene from pΔFBgus(R)-polyhedrin into the *polyhedrin* locus by Tn*7*-mediated transposition. The v*Ac23*null$^{/GUS+Ac23+PH}$ (*Ac23*null-repair) virus is the control of the *Ac23*null mutant virus as it contains the same *CAT* replacement of *Ac23* gene as that of the *Ac23*null virus. The *Ac23* gene, however, was reintroduced into the *polyhedrin* locus of the *Ac23* knockout bacmid along with a *GUS* reporter gene and a *polyhedrin* gene using Tn*7*-mediated transposition. Genotypes of the viruses were confirmed by PCR analysis. Two viruses carrying the *Ac23-GFP* transgene were generated for examination of *Ac23* localization by confocal microscopy. The *Ac23GFP* fusion gene was PCR amplified from pH5*Ac23* (a gift from Dr. Rollie Clem, Kansas State University) and cloned into PCR-II blunt vector (Invitrogen) to generate p*Ac23*GFP-PCRII-blunt. The PCR derived region was confirmed by sequencing. The *Ac23GFP* gene was subsequently cloned into the vector pΔFB-gus(R)-*polyhedrin* using BamHI and HpaI sites to generate the shuttle vector pΔFB-gus(R)-*Ac23GFP-polyhedrin*. The *Ac23-GFP* gene under the control of the *Ac23* promotor was then introduced into an *Ac23*null bacmid, and an *Ac23*-containing bacmid by Tn-7 mediated transposition. The recombinant bacmids were used to generate two *Ac23-GFP* fusion gene carrying viruses v*Ac23*null/$^{GUS\textit{Ac23}GFP+PH}$ (*Ac23*null-*Ac23GFP* repair) and v*Ac*BAC+/$^{GUS\textit{Ac23}GFP+PH}$ (*Ac*bacmid-*Ac23GFP*).

OBs were produced by infecting Sf9 cells (9 x 10^6 cells/T_{150} flask) with budded virus at a

multiplicity of infection of five. At seven days post infection, OB were purified from infected cells as described by O'Reilly *et al*. (1994), re-suspended in sterile water, and stored at 4°C. Prior to use, the OB suspension was gently vortexed for 20 min at room temperature to break up any aggregates that may have formed.

3.2.2 Scanning Electron Microscopy (SEM)

OBs were first captured by passing OB suspensions through a 0.4 μm polycarbonate filter (Nuclepore, Kent, WA), fixed for 1 hr at room temperature with 500 μL of modified Karnovsky's buffer (2.5% glutaraldehyde, 4% paraformaldehyde, 0.1 M sodium cacodylate buffer, pH 7.2) and then washed with 0.1 M sodium cacodylate buffer, pH 7.2 for 10 min at room temperature. OBs were then dehydrated at room temperature using 500 μL of each of the following graded ethanol series for 5 min each: 50%, 70%, 85%, 90%, and 100%. The ethanol was then removed and replaced with 500 μL of a 50:50 mixture of hexamethyldisilazane (HMDS) and absolute ethanol for 10 min, and then twice for 10 min each with 100% HMDS. The filters were then air-dried inside a fume hood for 1 hr and placed on aluminum mounts (Ted Pella, Inc., Redding, CA) using carbon tags (Marivac Limited, Halifax, NS). The samples were sputtered coated using a Polaron E5100 series II Sputter Coater (BioRad, Hercules, CA) and then photographed at 6000 x magnification using a Hitachi S-500 scanning electron microscope. Two perpendicular measurements (L1 and L2) across the widest region of each OB structure were taken as a measure of OB sizes.

3.2.3. Transmission Electron Microscopy (TEM)

Sample preparation for TEM was performed as described by Hong *et al.* (1994). OBs were pelleted at 14,100 x g for 8 min in a mini-spin microfuge (Eppendorf, Bristol, CT) and fixed for 1 hr in modified Karnovsky's buffer. Fixed OB were pelleted and encapsulated in 20 µl of 4% (w/v) low melting point agarose. The agarose block was washed with 0.1 M sodium cacodylate, pH 7.2 for 30 min at 4^{o}C and then incubated in a post-fixing solution (1% osmium tetroxide, 0.1 M sodium cacodylate, pH 7.2) for 1 hr at room temperature. The fixed samples were washed again and dehydrated at room temperature with the following graded ethanol series: 50%, 70%, 85%, 90% for 15 min each, 95% for 1 hr, and twice with 100% ethanol for 30 min each. Samples were then infiltrated with 25%, 50%, and 75% Spurr's resin/ethanol series (v/v, 12 hours each step). Final infiltration was performed in 100% Spurr's epoxy resin at room temperature for 30 min, followed by an overnight incubation (~16 hr). The resin was then polymerized in a 60°C oven for approximately 24 hr. Sections were cut with a Reichert Om U3 ultramicrotome at various thicknesses and mounted on either copper slot (1 x 2 mm) grids containing formvar and carbon film (serial sections), or 200 mesh copper grids (non-serial sections). All sections were stained in 5% uranyl acetate (10 min) followed by Reynolds lead citrate (1 min) before examining and photographing using an Hitachi H-600 TEM operated at 75kV.

A. Serial sections of viruses' occlusion bodies.

Serial sections of 100 nm thickness (*Ac*MNPV E2 and *Ac*bacmid) and 200 nm thickness (*Ac23*null mutant virus) were cut and photographed at 6000 x magnification (*Ac*MNPV

E2 and *Ac*bacmid) and 5000 x magnification (*Ac23*null mutant). Independent OBs were identified, numbered and followed through consecutive sections. The longest diameter (L1) in the section containing the widest OB was measured from the micrograph. The longest perpendicular measurement relative to L1 was taken (L2) and the average of L1 and L2 was used as a measure of the size of the OB. The number of ODV particles in each widest OB section was also determined.

B. Non-serial sections of *Ac23*null mutant and control virus occlusion bodies.

Individual 100 nm thick sections of the four viruses were examined by TEM and photographed at 10,000 x magnification for determination of the number of nucleocapsids in each ODV. To ensure ODVs observed in the individual sections were truly independent, every fifth section was kept for analysis and intervening sections were discarded. Only transverse sections of ODVs that were well stained and had a clear envelope were chosen for nucleocapsid calculation. ODVs that clearly had more than five nucleocapsids, but for which the exact number could not be accurately determined, were grouped into a separate class.

3.2.4. Confocal Microscopy

To examine *Ac23* localization by confocal microscopy, Sf9 cells were seeded into glass bottom culture dishes (9×10^5 cells per dish) (MetTek, Ashland, MA) and incubated for 1 hr at 27°C. Cells were then either mock infected, infected with a GFP-minus control virus (*Ac23*null-repair), or infected with viruses carrying *Ac23-GFP* transgenes (*Ac23*null-*Ac23GFP* repair and *Ac*bacmid-*Ac23GFP*). At 48 and 77 hours post infection,

medium was removed and cells were fixed with 10% formalin for two min, washed with PBS once, stained with 300 nM DAPI (Invitrogen, Carlsbad, CA) in PBS for three min, and rewashed three times with PBS. Cells were observed with a Nikon C1+ confocal microscope using a 405 diode laser for DAPI and an Argon 488 laser for GFP. Differential interference contrast (DIC) imaging was used to provide contrast to the unstained cells. All images were captured using X60/1.2 N.A. water immersion lens.

3.2.5. Statistical Methods

Data analysis was done using SPSS 15.0 software (SPSS Inc. Chicago, IL). Statistical analysis consisted of determinations of mean and standard deviation of viral particle size. Pearson correlation coefficient was used to test the trend for an association between the number of ODV particles and the size of the widest OB section. A simple linear regression model was applied to evaluate the prediction of ODV numbers from the size of the widest OB section. Group differences were compared by ANOVA test and Tukey test. All *p* values were determined by two-tailed test and *p* values less than 0.05 was regarded as statistically significant.

3.3 Results

3.3.1. OBs from bacmid-derived viruses are smaller than those of wildtype viruses

During previous OB quantitation by light microscopy, it was observed that OBs from *Ac23*null viruses appeared smaller than OBs from control *Ac*MNPV (unpublished observation). In the present study we made detailed measurements of OB size using both

SEM and TEM. A schematic diagram of the genetic differences between the *Ac23*null virus and three control viruses used in the present study is presented in Figure 3.1. Since OBs are polyhedral crystals, I used the average of the two largest perpendicular length measurements as a measure of OB size. SEM analysis showed that OBs from all four groups of viruses showed intra-group size variation (Fig. 3.2). Significant size differences were found between wildtype (*Ac*MNPV E2) OBs and OBs from the bacmid-derived viruses using the ANOVA test and Tukey test (Fig. 3.3, $p<0.0001$). Wildtype OBs are generally larger than OBs of the *Ac23*null-repair virus (mean ± SD, 2.48 ± 0.60 vs. 1.62 ± 0.44 μm, $p<0.0001$), the bacmid-derived control *Ac*bacmid (2.48 ± 0.60 vs. 1.74 ± 0.44 μm, $p<0.0001$) and the *Ac23*null mutant (2.48 ± 0.60 vs. 1.75 ± 0.54 μm, $p<0.0001$). However, there was no significant difference between the sizes of OB from the two bacmid-derived control viruses (*Ac23*null-repair and *Ac*bacmid), and between the bacmid-derived control viruses and the *Ac23*null virus using the ANOVA test and Tukey test. The sizes of OBs were also determined by measuring the widest OB cross section found in TEM serial sections of purified agarose-embedded OB. Consistent with the SEM analysis, statistically significant size differences were observed between the OB sizes of *Ac*MNPV E2 and the bacmid-derived viruses (*Ac23*null and *Ac*bacmid, Fig. 3.3). The mean size of the widest section of *Ac*MNPV E2 OBs are greater than that of *Ac*bacmid (2.39 ± 0.65 vs. 1.87 ± 0.41 μm, $p < 0.0001$) and *Ac23*null mutant (2.39 ± 0.65 vs. 1.71 ± 0.46 μm, $p < 0.0001$). Thus, both SEM and TEM analysis indicate that bacmid-derived OBs were on average smaller than those from wildtype *Ac*MNPV E2, from which the bacmid was derived. However, the size of OBs from the *Ac23*null mutant is not significantly different from the size of OBs from bacmid-derived *Ac23*-containing

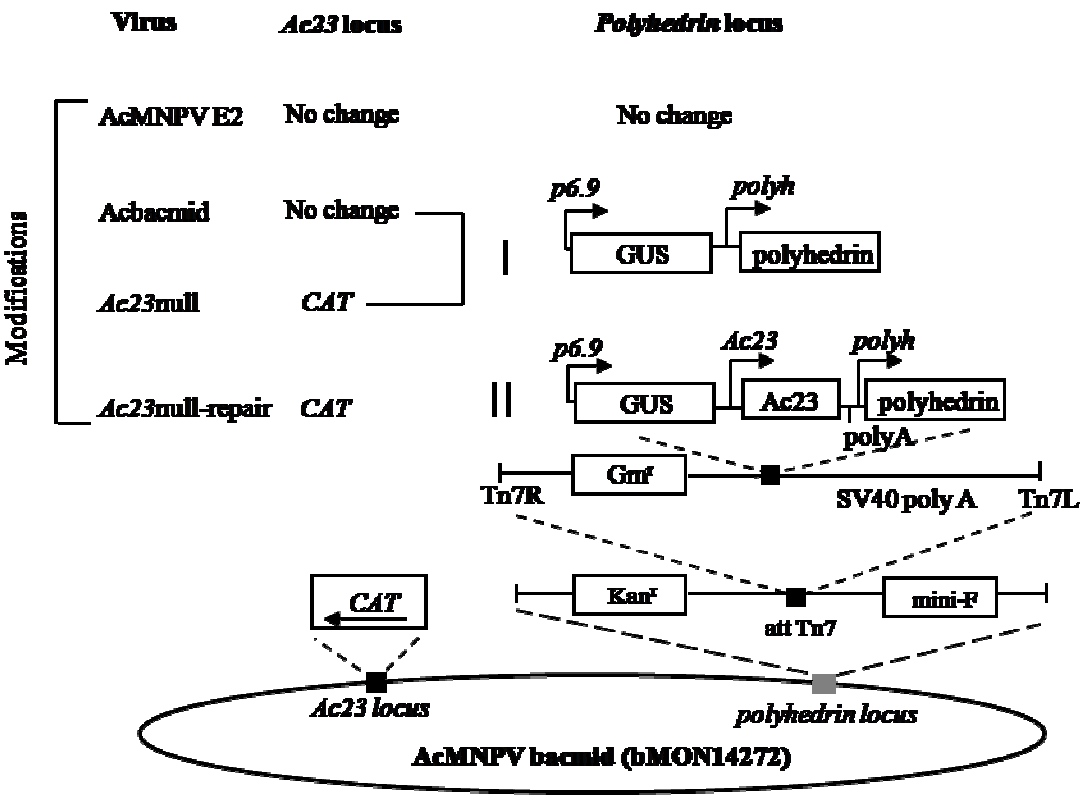

Fig. 3.1 Diagram comparing the differences between the *Ac23*null virus and three control viruses. The differences occur at the *Ac23* locus and the *polyhedrin* locus. There are no modifications to the *Ac23* locus in the *Ac*MNPV E2 virus and the *Ac*bacmid virus, but the *Ac23* gene in both *Ac23*null and *Ac23*null-repair was replaced by a *CAT* gene. There are no modifications in the *polyhedrin* locus of the *Ac*MNPV E2 virus, but all three bacmid-derived viruses (*Ac*bacmid, *Ac23*null, *Ac23*null-repair) are *polyhedrin* gene-negative due to replacement of the *polyhedrin* gene with a cassette containing the mini-F replicon, a kanamycin resistance gene, and a mini-attTn*7* site for accepting Tn*7* mediated transposition (Luckow, 1993). Two types of transfer vectors were used to insert genes into the *polyhedrin* locus by Tn*7* mediated transposition. Type I vectors were used to

insert genes into the *Ac*bacmid and *Ac23* null bacmid, and contained a *GUS* reporter gene under the control of a P6.9 promoter and a *polyhedrin* gene under the control of its own promoter. Type II vector was used to generate the *Ac23*null-repair bacmid, and contained an *Ac23* gene under the control of its own promoter in addition to the *GUS* and *polyhedrin* genes found in the type I vector. Abbreviations: polyh = polyhedrin promoter; Gm^r = Gentamycin resistance marker; attTn*7* = Tn*7* attachment site.

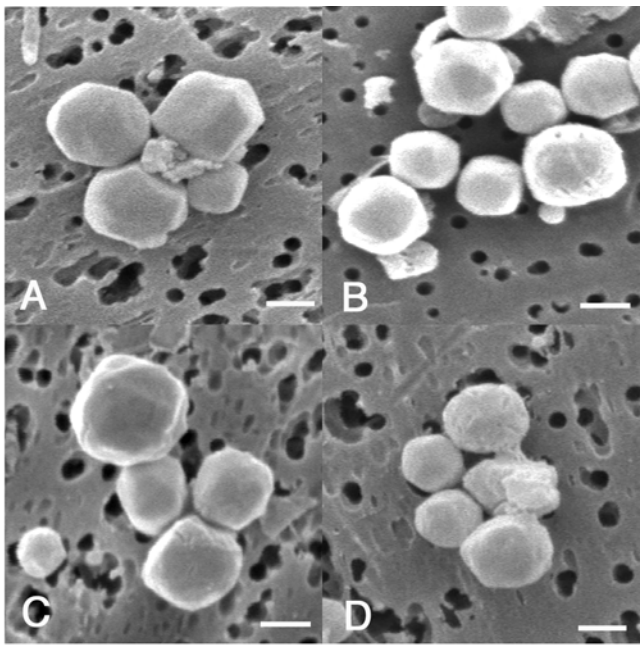

Fig. 3.2 Scanning electron micrographs of occlusion bodies of A) *Ac*MNPV E2, B) *Ac*bacmid, C) *Ac23*null-repair, and D) *Ac23*null viruses. Scale bars = 1µm.

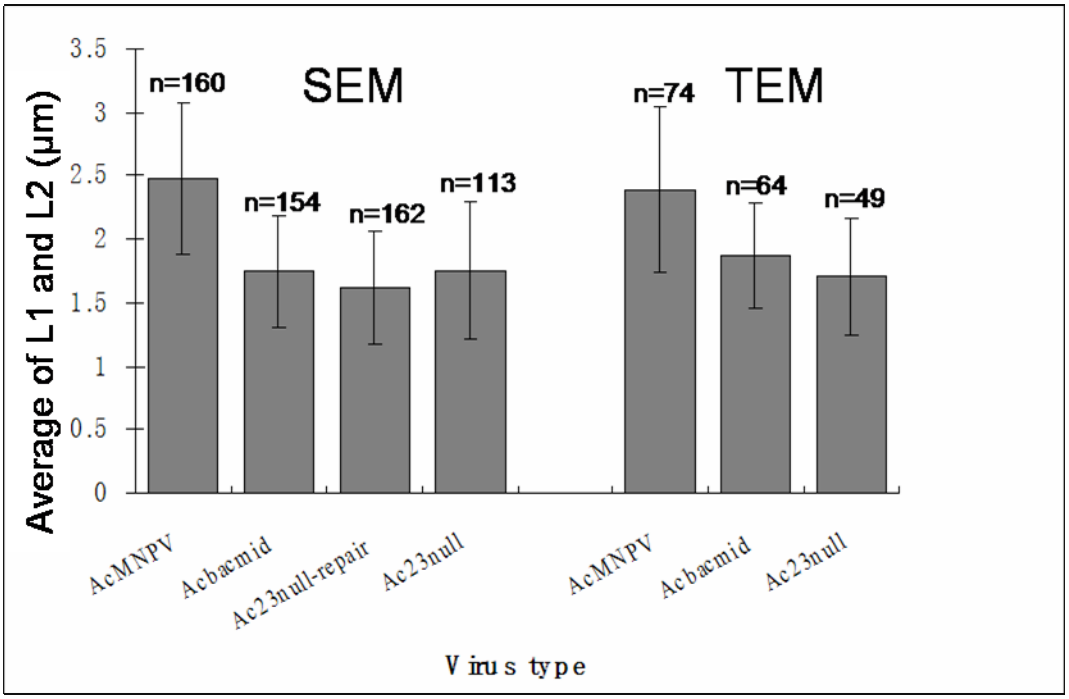

Fig. 3.3 Comparison of occlusion body size by analysis of scanning electron micrographs (SEM) and transmission electron micrographs (TEM). Each bar represents the average of L1 and L2 OB diameters, and the error bars represent the standard deviation. Significant size differences were found between wildtype OBs and OBs from the bacmid-derived viruses using the ANOVA test and Tukey test ($p<0.0001$). No significant differences in the sizes of OB were found between *Ac*bacmid, *Ac23*null-repair and *Ac23*null mutant.

control viruses.

3.3.2. A significant and positive relationship was found between the number of ODV and the widest section of OB.

To examine whether the number of occluded ODVs within OBs differed between *Ac23*null and control viruses, the section with widest diameter of 47 *Ac*MNPV E2 OBs, 37 *Ac*bacmid OBs, and 26 *Ac23*null mutant OBs were used to determine the number of ODVs present in the section. The number of ODVs per widest dimension of OB serial section varied between different OBs of the same genotype. However, the average ODV numbers in the widest OB sections were similar among the three types of viruses (*Ac23*null: 18.58 ODVs/section, SD=12.51; *Ac*bacmid: 18.68 ODVs/section, SD=10.5; *Ac*MNPV E2: 19.96 ODVs/ section, SD=11.08). The number of ODV particles within the section is positively correlated with the size of the widest OB section for all three viruses (Pearson correlation coefficient: *Ac23*null virus r = 0.50, p = 0.007; *Ac*bacmid r = 0.37, p = 0.024; *Ac*MNPV E2 r = 0.51, p =0.0002). The size of the widest OB section was a significant and positive predictor of ODV number (Fig. 3. 4) in *Ac23*null (mean ± SE, $\beta = 14.74 \pm 5.06$, p = 0.007), *Ac*bacmid ($\beta = 10.23 \pm 4.28$, p = 0.02), and AcMNPV E2 ($\beta = 8.94 \pm 2.24$, p = 0.0002) virus. The goodness of fit (R^2) of the regression model was 25.4% for *Ac23*null, 14.0% for *Ac*bacmid, and 26.2% for *Ac*MNPV E2. Extreme examples of the widest section of two different similarly sized OBs with dramatically different ODV numbers are shown in Fig. 3.5. This variability may be partially explained by the low R^2 of the regression model. Determining the exact number of ODVs present in an entire OB by examination of serial section series was not possible because of the

reduced resolution and contrast due to the formvar and carbon films necessary for serial section support. However, we consistently find that ODV abundance is comparable throughout the serial section series of any particular OB with the exceptions of the ends. These results suggest that the ODV number calculated in the widest OB section is a good indicator of the abundance of ODVs in a particular OB, and there are likely no significant differences on average between the ODV number of *Ac23*null OBs and *Ac*bacmid control OBs.

3.3.3. Nearly forty-five percent of *Ac23*-null mutant ODV contains a single nucleocapsid.

Since no significant difference was found in the size and ODV content of *Ac23*null OBs and the bacmid-derived control OBs, we examined whether nucleocapsid numbers within *Ac23*null mutant ODVs differed from ODVs produced by *Ac23*-containing viruses. Transverse sections of a total of 337 *Ac*MNPV E2 ODVs, 376 *Ac*bacmid ODVs, 326 *Ac23*null-repair ODVs, and 289 *Ac23*null mutant ODVs were analyzed to determine the number of nucleocapsids enclosed within them using electron micrographs (Table 3.1 and 3.2). The results show a significant difference in the nucleocapsid numbers found within ODV particles of the different genotypes. A very high percentage (44.6%) of *Ac23*null mutant ODVs contains a single nucleocapsid. In contrast, the percentage of single nucleocapsids in the controls, *Ac23*null-repair ODV (11.3%), *Ac*bacmid ODV (21.8%) and *Ac*MNPV E2 (13.6%) ODV are significantly lower. The opposite trend was observed when the percentages of ODV containing five or more nucleocapsids were compared (*Ac23*null mutant, 17%; *Ac23*null-repair 49.7%; *Ac*bacmid, 39.4%; *Ac*MNPV E2, 55%,

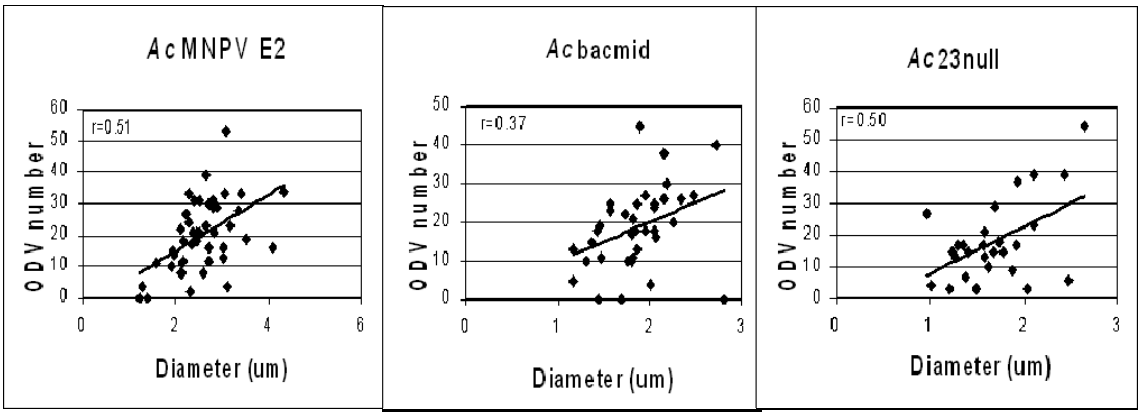

Fig. 3.4 Scatter plot of occlusion body sizes versus ODV numbers of A) *Ac*MNPV E2, B) *Ac*bacmid, and C) *Ac23*null viruse. The data graphed represents a subset of the widest OB sections, which ODV number can be accurately determined.

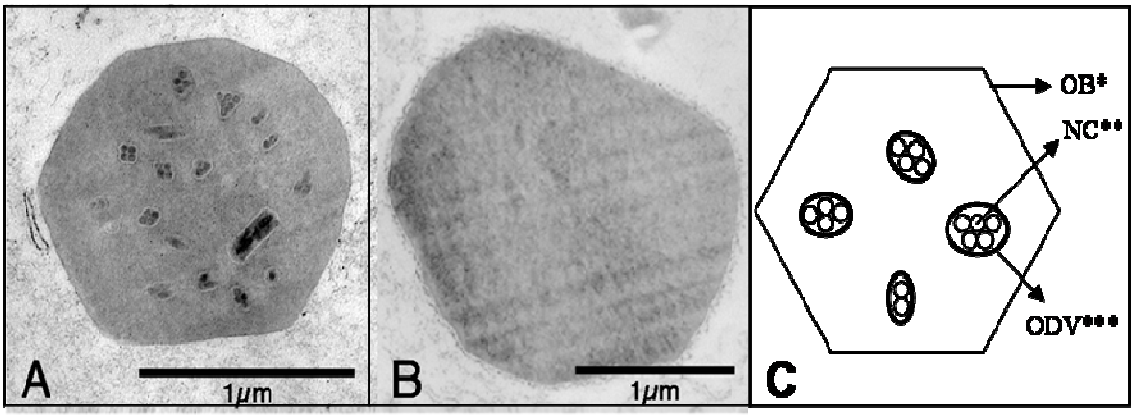

Fig. 3.5 Extreme examples of two different wildtype *Ac*MNPV OBs showing the variations between OB size and ODV number. A) The widest serial section of OB with 17 ODV, and B) the widest serial section from a different OB with no ODV, C) a schematic diagram illustrating the relationship between OB and its components.

Table 3.1). The maximum nucleocapsid number that can be clearly distinguished in an *Ac23*null mutant ODV on micrographs was seven (1.8% of the total *Ac23*null ODV examined), however, ODVs with up to fifteen clearly distinguishable nucleocapsids can be observed in all three types of *Ac23* containing viruses (Table 3.2). Very rarely, ODVs of *Ac23*null and *Ac23* containing viruses with more than 15 nucleocapsids were observed in the sections with the electron microscope (data not shown), however the exact nucleocapsid number in such ODVs cannot be determined from the micrographs. Consistent with the result that *Ac23*null ODVs contained mostly single or double nucleocapsids, a higher percentage (43%) of *Ac23*null OB sections on the micrographs only contained ODVs with nucleocapsid numbers of three or less. In contrast, 23.4%, 30.96% and 14.58% of the *Ac23*null-repair, *Ac*bacmid and *wt Ac*MNPV OB sections examined contained only ODVs with 1-3 nucleocapsids, respectively. Thus, since the ODV number within the widest OB section are not significantly different among all viruses OB, the data suggest that *Ac23*null OBs contain significantly fewer nucleocapsids per OB than *Ac23*-containing control OBs.

Table 3.1 Frequency distribution of nucleocapsid number enclosed within *Ac23*null mutant and control virus ODV.

	nucleocapsid number				
Virus	1	2	3	4	5 or more
*Ac23*null ODV (n=289)	44.6% (129)	14.2% (41)	12.1% (35)	12.1% (35)	17% (49)
*Ac23*null-repair ODV (n=326)	11.3% (37)	15.3% (50)	12% (39)	11.7% (38)	49.7% (162)
*Ac*bacmid ODV (n=376)	21.8% (82)	14.6% (55)	14.4% (54)	9.8% (37)	39.4% (148)
*Ac*MNPV E2 ODV (n=337)	13.6% (46)	8.3% (28)	8.6% (29)	14.5% (49)	55% (185)

Table 3.2 Frequency distribution of nucleocapsids in ODV that contain 5 or more nucleocapsids.

Virus	capsid number												unclear 5
	5	6	7	8	9	10	11	12	13	14	15	16	or more*
*Ac23*null (n=49)	3.1% (9)	2.4% (7)	1.7% (5)	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)	9.7% (28)
*Ac23*null-repair(n=162)	6.7% (22)	4% (13)	3.7% (12)	1.5% (5)	0.3% (1)	0.9% (3)	0.6% (2)	0% (0)	0% (0)	0% (0)	0% (0)	0.3% (1)	31.6% (103)
*Ac*bacmid (n=148)	8.8% (33)	1.9% (7)	2.7% (10)	0.8% (3)	0% (0)	1.1% (4)	0.8% (3)	0.3% (1)	0.3% (1)	0% (0)	0.3% (1)	0.3% (1)	24.2% (91)
*Ac*MNPV E2 (n=185)	4.5% (15)	5% (17)	1.8% (6)	1.8% (6)	0.6% (2)	0.6% (2)	0.6% (2)	0% (0)	0.3% (1)	0% (0)	0.3% (1)	0% (0)	39.5% (133)

*nucleocapsid number greater than five, but the exact number can not be determined

3.3.4. Nucleocapsid distribution pattern.

The rod-shaped ODV nucleocapsids are typically bundled together lengthwise and examination of a large number of ODV cross sections on the micrographs show that the majority of ODVs with fewer than 11 nucleocapsids have a specific nucleocapsid arrangement pattern (Fig. 3.6). Nucleocapsids in two-nucleocapsid ODVs are always arranged side-by-side (Fig. 3.6B, n=174); when a third nucleocapsid is present, the nucleocapsids are always arranged in a 1-2 triangular pattern (Fig. 3.6C, n=157); when four nucleocapsids are present, the nucleocapsids always form a 1-2-1 rhombus pattern (n=159). Most of these rhombus-shaped ODVs form lozenges (Fig. 3.6D, n=137), but some appear rounder and are more like squares (n=22). When five nucleocapsids are present these nucleocapsids usually form a 2-3 isosceles trapezoid and appear to be modified from a four nucleocapsid lozenge (Fig. 3.6E, n=73), while a small percentage forms a 1-2-2 irregular pentagon that appears to be modified from a four nucleocapsid square (n=6). In six nucleocapsid ODVs, the nucleocapsids almost always form a 1-3-2 irregular pattern (Fig, 3.6F, n=43), while a very small percentage formed a 1-2-3 equilateral triangle (n=1). Seven nucleocapsid ODVs always form a 2-3-2 hexagon (Fig. 3.6G, n=33). Eight nucleocapsid ODVs form a 1-2-3-2 irregular pattern (Fig. 3.6H, n=14), which appears to be a seven nucleocapsid ODV with an additional nucleocapsid positioned on one side of the seven-nucleocapsid hexagon. Among three ODVs with nine nucleocapsids found on the micrographs, two of them formed an irregular 2-4-3 pattern (Fig. 3.6I), while the other formed an irregular 3-3-3 pattern. Both of these patterns have a concave spot that can be filled by a tenth nucleocapsid to form the same 3-4-3 regular hexagon pattern (Fig. 3.6J, n=9). In eleven-nucleocapsid ODV's, the

eleventh nucleocapsid is positioned either along the longer side of the hexagon (Fig. 3.6K, n=6) to form a 3-4-3-1 irregular pattern, or the shorter side of the hexagon to form a 3-4-4 irregular pattern (n=1). From the electron micrographs, relatively few ODVs examined have more than eleven nucleocapsids that can be determined precisely (Fig. 3.6L-O, n=7). Additional patterns of 5, 6, 8, 9, 11 nucleocapsids ODVs were occasionally observed in sections under EM (data not shown). No differences in nucleocapsid arrangement patterns were observed between ODVs from *Ac23*null and ODVs from control viruses.

3.3.5. *Ac23*-GFP localizes to the nuclear membrane.

Two recombinant baculovirus carrying a *Ac23-GFP* transgene were used to examine the localization of *Ac23* in infected cells by confocal microscopy. As expected for an envelope protein that is present on BVs, cells infected with the *Ac23*-GFP expressing viruses showed *Ac23*-GFP fluorescence at the plasma membrane and the cytoplasm of many infected cells (Fig. 3.7). However, *Ac23*-GFP fluorescence also showed strong localization at the nuclear envelope of infected cells and in many cases, the nuclear envelope localization at 48 hpi is much more prominent than localization at the plasma membrane (see Fig. 3.7). Since the nuclear envelope is the most likely source of ODV envelope, the localization of *Ac23*-GFP to the nuclear envelope is consistent with *Ac23* playing a role in the envelopment of ODV nucleocapsids.

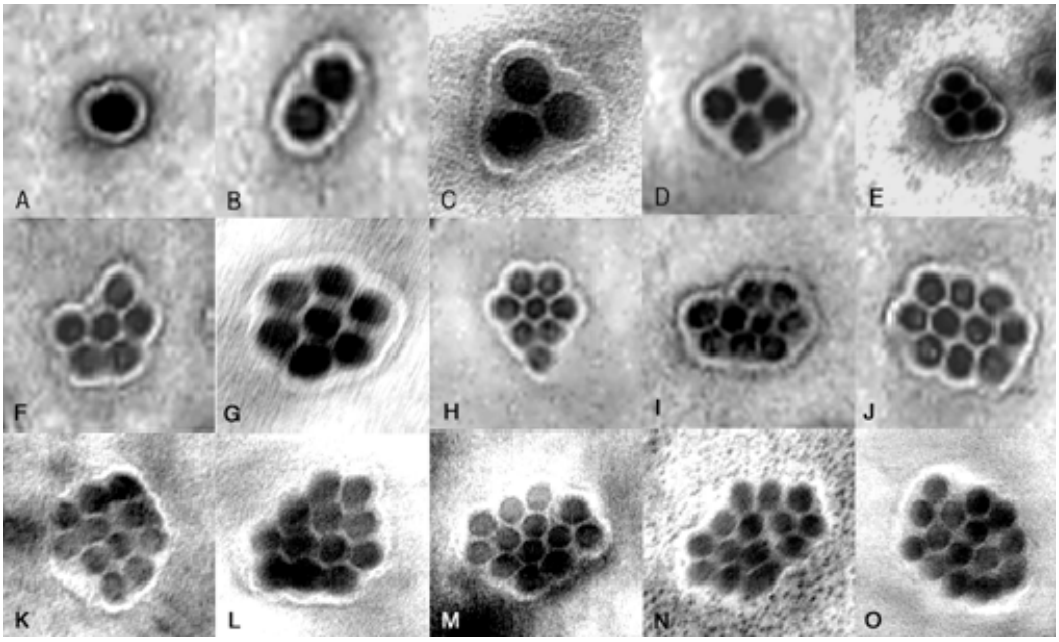

Fig. 3.6 Nucleocapsid organization in ODV containing different numbers of nucleocapsids. Representative example of transverse sections of ODV containing A) 1, B) 2, C) 3, D) 4, E) 5, F) 6, G) 7, H) 8, I) 9, J) 10, K) 11, L) 12, M) 13, N) 14, and O) 15 nucleocapsids.

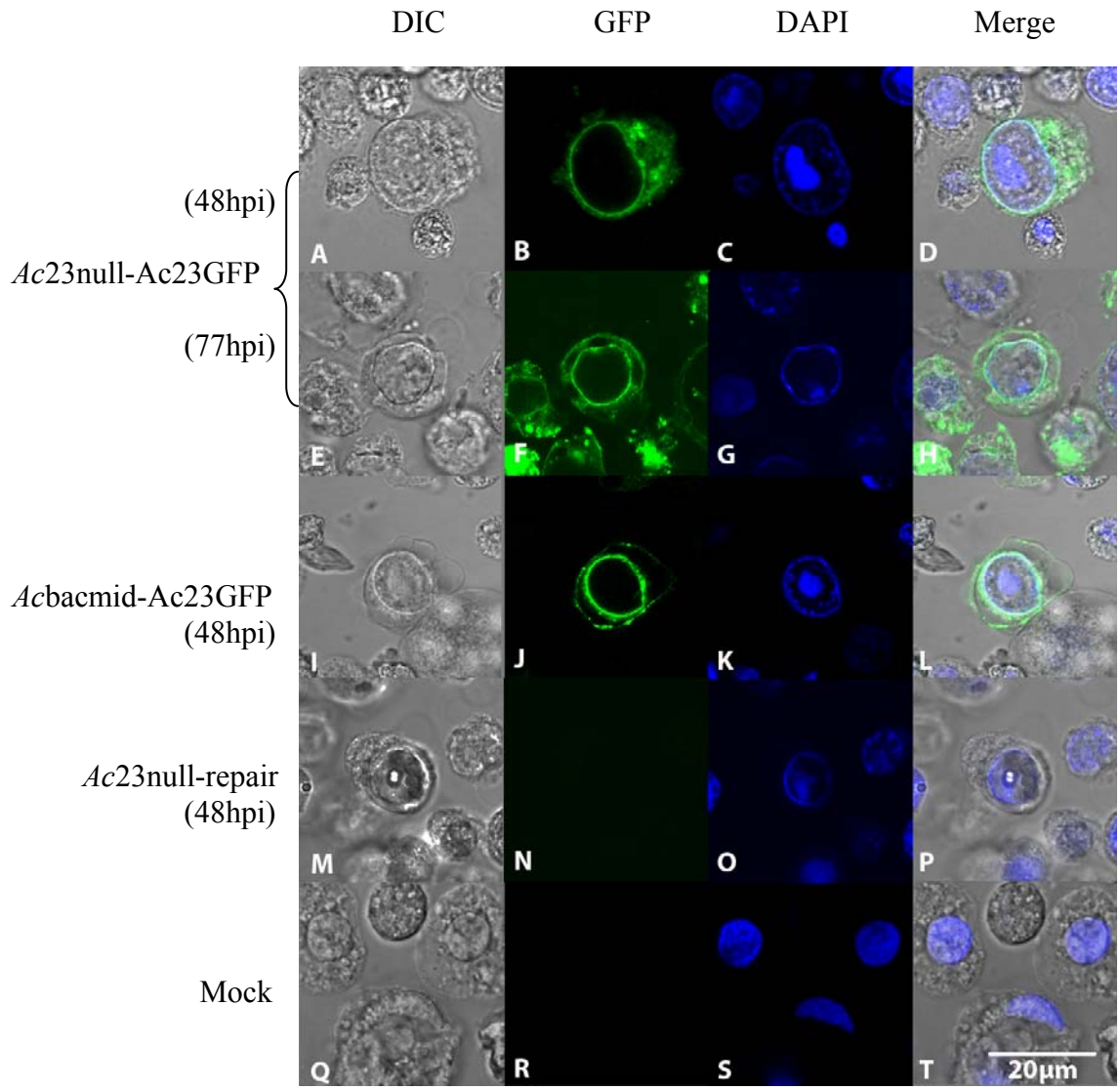

Fig. 3.7 *Ac23*-GFP protein localizes to the nuclear envelope of infected cells. Confocal images of Sf9 cells that were infected with either of two *Ac23*-GFP expressing viruses: *Ac23*null-*Ac23GFP* repair virus (48 hpi: A - D; 77 hpi: E - H), and *Ac*bacmid-*Ac23GFP* virus (I – L, 48 hpi), or infected with a control GFP-minus virus *Ac23*null-repair (M – P, 48 hpi), or mock infected (Q - T). DIC images are shown in panels A, E, I, M, Q; GFP

fluorescence is shown in panels B, F, J, N, R; DAPI fluorescence is shown in panels C, G, K, O, S; and images of all three channels merged are shown in panels D, H, L, P, T.

3.4 Discussion

Two different types of envelope fusion proteins (GP64 and F) have been identified in the baculovirus. Analysis of sequenced baculovirus genomes (Hiscock *et al.,* 2000) revealed that a small number of closely related baculovirus (group I NPVs) have both *gp64* gene, and an *f* gene; most baculovirus (group II NPVs, GVs and a *Diptera* NPV) contain no *gp64* gene, but have an *f* gene; and hymenopteran baculovirus (*Neab*NPV, *Nese*NPV, and *Nele*NPV) do not appear to have either gene (Duffy *et al.,* 2006; Garcia-Maruniak *et al.,* 2004; Lauzon *et al.,* 2004). The presence of *f* gene homologs in viruses with a functional *gp64* fusion protein gene such as *Ac*MNPV and *Op*MNPV is intriguing, especially since the F homologs in *Ac*MNPV (Ac23) and *Op*MNPV (Op21) do not have detectable membrane fusion activity in membrane syncytium assays (Lung *et al.,* 2003; Pearson *et al.,* 2000). Pearson and Rohrmann (2002) proposed that *f* genes encode the prototypical BV envelope fusion protein and that group I NPVs acquired the *gp64* gene more recently. The acquisition of *gp64* subsequently displaced the membrane fusion function of the F protein in the group I NPVs. The lack of membrane fusion activity in Ac23 and Op21 may be due to the absence of a furin-cleavage site, which has been shown to be essential for membrane fusion activity of the *Se*MNPV F protein (Lung *et al.,* 2003; Westenberg *et al.,* 2002). Characterization of an *Ac23*null mutant *Ac*MNPV virus showed that this gene is not essential for viral infectivity in cultured cells or in animals (Lung *et al.,* 2003). However, *Ac23*null mutant OBs had a slower speed of kill. Results presented in the

current study show that the *Ac23*null mutant OBs were on average: 1) similar in size to bacmid-derived control OBs, 2) had comparable numbers of ODVs per OB as the control OBs, and 3) contained a significantly higher percentage of ODVs with single nucleocapsid (44.5% in the null mutant vs. 11.3% in the near isogenic control). These results suggest that *Ac23*null OBs contain ODVs with fewer nucleocapsids than control OBs. Detailed analysis of nucleocapsid arrangement in ODVs in this study suggests that the spatial organization of nucleocapsids is similar between ODVs from *Ac23*null and control viruses. The spatial organization of nucleocapsids within *Ac*MNPV ODVs with 1, 2, 3, 7, and 10 nucleocapsids are similar to those described for *Euproctis similes* NPV (*Eusi*NPV) (Kawamoto *et al.,* 1974). Nucleocapsid arrangement patterns not described by Kawamoto and Asayama for *Eusi*NPV were observed in *Ac*MNPV ODVs with 4, 5, 6, 8, 9 and 11 nucleocapsids.

When ODVs fuse with midgut cells, their nucleocapsids are transported to the nucleus where they unpackage their genomes and begin viral replication. Progeny virus nucleocapsids are produced in the nucleus and are transported to the basolateral side of the cell membrane where they bud out as BVs. When multiple ODV nucleocapsids are delivered to midgut cells an alternate pathway of infection may occur (Adams *et al.,* 1977; Granados *et al.,* 1981). In the alternate pathway some ODV nucleocapsids bypass the nucleus and migrate directly to the basolateral membrane. Other ODV nucleocapsids enter the nucleus, unpackage their genomes and express *gp64* which has an early promoter motif (Blissard and Rohrmann, 1991). GP64 proteins are transported to the basolateral membrane and enable ODV-derived nucleocapsids to bud out as BV. This

alternative pathway accelerates the progression of infection in the host by many hours (Adams *et al.,* 1977; Granados *et al.,* 1981). Animals infected with *Ac23*null mutant OBs may have survived longer than those infected with control OBs in part because *Ac23*null viruses had fewer ODV nucleocapsids for the faster alternative pathway of infection. The packaging of multiple nucleocapsids into virions is a hallmark of MNPVs, and has been suggested to offer a selective advantage over baculovirus that package a single nucleocapsid per virion (SNPV) (Washburn *et al.,* 1999, 2003). Recently, it was reported that *Ac142* is essential for nucleocapsid evelopment to form ODV (McCarthy *et al.,* 2008). The observation in this study that *Ac23*null ODVs with high nucleocapsid counts are present, and that the percentage of *Ac23*null ODVs with 2 – 4 nucleocapsid counts are comparable to the controls suggests that Ac23 is not essential for multiple nucleocapsid envelopment, but it might somehow facilitate multiple nucleocapsid envelopment.

Consistent with Ac23 playing a role in the envelopment of ODV nucleocapsids, Ac23 was recently identified as a component of the ODV, and proposed to be an ODV envelope protein (Braunagel *et al.,* 2003). The source of the ODV envelope has not yet been conclusively demonstrated, but the nuclear membrane is considered a likely source. Thus, my observation that Ac23 localizes to the nuclear membrane is consistent with Ac23 being not only a BV envelope protein, but also an ODV envelope protein, and it may plays a role in the envelopment of ODV nucleocapsids. Conceptual translation of the Ac23 protein sequence reveals the presence of a putative nuclear localization signal (PKKKFNF) at position 513-519 of the protein that may be involved in nuclear envelope localization. Ac23 also has a putative long C-terminal tail that could play a role in ODV

nucleocapsid envelopment by physically interacting with ODV nucleocapsid components (data not shown). Whether Ac23 interacts with the major capsid protein VP39 or other capsid associated proteins such as p80, p24, and/or orf1629 (pp78/83) to influence ODV nucleocapsid envelopment remains to be determined. Since Ac23 has also been shown to be associated with BV (Lung *et al.,* 2003), and since most BVs contain single nucleocapids, factors in the BV membrane (e.g. GP64), BV nucleocapsid or the cytosol/plasma membrane may inhibit Ac23 promotion of multiple capsid packaging in BVs. It should however be noted that multiple capsid *Ac*MNPV BVs have been observed under EM (Adams *et al.,* 1991). Alternatively, unidentified factors found exclusively in the ODV membrane, ODV nucleocapsid or the nucleus could be enabling Ac23 to promote envelopment of multiple nucleocapsids into the ODV. In SNPVs, the F homologues and/or their interaction partners may have enough sequence divergence to prevent the envelopment of multiple capsids into a single ODV. Consistent with this hypothesis, F homologues from even the most closely related group I NPVs with sequence available are poorly conserved. For example, amino acid identity between the mature (without the predicted signal sequence) Ac23 and the Op21 protein from *Op*MNPV is only approximately 47.9%, while the percent identity between mature Ac23 and Bm14 from *Bm*NPV (an MNPV) is only 37.6%. Single step growth curve analysis performed in Sf9 cells suggests that the *Ac23*null mutant BV have slightly slower growth kinetics in the first 24 hr post infection when compared to control viruses (Lung *et al.,* 2003). In both Sf9 and High-5 cells, and at all time points examined, progression of infections in *Ac23*null BV infected cells is slower than control BV infected cells. These observations indicate that *Ac23* also enhance BV infection, perhaps by promoting binding

to cell surface receptors and facilitating viral entry.

In GP64 containing viruses, it appears that F protein has been retained to perform multiple auxiliary functions. Ac23 appears to play distinctive roles in the BV and in the ODV. Generation of ODVs with higher nucleocapsid numbers could facilitate rapid establishment of primary infection, while facilitating BV infection promotes progeny virus production, systemic infection and OB formation. In contrast, GP64 has been shown to be associated exclusively with BVs (Blissard *et al.,* 1989). These intriguing observations further support the contention that the F protein plays a different role from that of GP64 in viruses that contain both proteins. Whether Ac23 has an effect on BV or ODV binding to cell surface receptors or ODV fusion with midgut cells, in addition to its role in ODV assembly, remains to be determined.

F protein is likely to have evolved in baculovirus genomes over a longer period of time than the more recently acquired GP64. Thus it is not surprising to discover that F proteins may have evolved pleotrophic functions beyond envelope fusion. My data suggest that although *Ac23* is a nonessential gene, its effects on nucleocapsid packaging in ODVs, and BV spread could offer significant selective advantages to *Ac*MNPV.

Conclusion

Enveloped viruses, such as baculoviruses, require fusion proteins on the viral envelope for cell recognition and entry. Two different types of envelope fusion proteins have so far been identified in baculoviruses. GP64 is a well characterized low pH-activated fusion protein that is found in a relatively small group of baculovirus (group I NPVs) in which *Ac*MNPV belongs. *Ac*MNPV is unique among baculoviruses in that it can enter a variety of cell types, including non-host cells such as human hepatocytes. This is likely due to the presence of GP64 on the virion. F (fusion) proteins are found in both group I and group II NPVs, but so far fusion activity have only been demonstrated for F proteins from group II NPVs.

In chapter two of this thesis I examined the significance of GP64 and the *Se*F (F protein from SeMNPV) in mediating viral entry into vertebrate cells, and evaluated the possibility of developing a *gp64*null *Ac*MNPV vector with minimal tropism for non-insect cells. The transduction efficiency of the genetically engineered *Ac*MNPV variants that either have the endogenous GP64 envelope protein, or the *Se*F protein, or have both GP64 and *Se*F were evaluated on 15 different vertebrate cell lines. I have shown that transduction efficiency of *Se*F-pseudotyped *gp64*null *Ac*MNPV on HepG2, BHK-21, PK-15 and FKC cells were at least 50 fold lower than that of the GP64-containing control viruses. Transduction efficiency for human HepG2 dropped dramatically from approximately 98% to approximately 0.15% when v*gp64nullAc*$^{Se\text{F-CMV-hrGFP}}$ was used in

place of v*gp64nullAc*$^{GP64\text{-}CMV\text{-}hrGFP}$. Except for the three fish-derived cell lines, transduction efficiencies of *Se*F-pseudotyped *gp64*null virus were lower than 1% on all cell lines tested. These results indicate that the tropism of *Ac*MNPV virus for vetebrate cells can be restricted by pseudotyping a *gp64*null virus with *Se*F. Thus, the *Se*F-pseudotyped *gp64*null vector may be a useful platform for incorporation of additional targeting molecules for targeted gene delivery. Low levels of transduction (0.2%) by the *Se*F-pseudotyped *gp64*null virus was observed with 4 mammalian cell lines. These low level transductions can be further eliminated by using a lower virus to cell ratio, or potentially also by using engineered *Se*F proteins that contain mutations such as truncations in the ectodomain. My results also indicated that GP64 is a critical factor for efficient transduction of vertebrate cells by *Ac*MNPV. Five recombinant bacmids with CMV-luciferase cassettes in place of the CMV-hrGFP cassettes were also generated, but have not been made into viruses and used in evaluation of transduction (described in materials and methods section of chapter 2). These reagents could be used in the future for a more quantitative examination of *Ac*MNPV-mediated gene expression in cell lines and for *in vivo* animal studies.

During the course of this study I also made the interesting observation that GP64 can mediate cell-cell fusion in the absence of low pH-activation in SCP and MDBK cells. To my knowledge this phenomenon has not been previously reported, and suggest there are alternative mechanisms (pH-independent) that can activate the membrane fusion activity of GP64. The biological significance of this phenomenon and the mechanism by which GP64 can be activated at neutral pH remains to be determined.

The function of the *Ac*MNPV F homolog (Ac23) is unclear, since Ac23 does not have detectable membrane fusion activity, and is not required for AcMNPV propagation and pathogenicity. The presence of this gene in the AcMNPV genome and the fact that it is expressed and incorporated into virions strongly suggest the *Ac23* gene or its gene product likely offer significant selective advantage to this virus. Previous reports have shown *Ac23* to be a pathogenicity factor that increases AcMNPV's speed of kill. However, the mechanism by which *Ac23* accelerates host death is unclear. Chapter 3 of this thesis describes a comparison of occlusion bodies (OB) and occlusion derived virus (ODV) made by an *Ac23*null mutant and those from control viruses. My results show that while OB size and the number of ODV per OB were comparable, 44.6% of *Ac23*null virus ODVs contain single nucleocapsid per ODV in comparison to the 11.3% found in the near isogenic control (*Ac23*null-repair) ODVs. This observation suggests that Ac23 could some how facilitate the incorporation of multiple nucleocapsids into each ODV particle. Since nucleocapsid number is equal to the genome copy number, *Ac23*null ODVs (the infectious unit of AcMNPV) contains less genome copies than viruses with the *Ac23* gene intact. This result suggest that Ac23 OBs and ODVs have fewer nucleocapsids than wildtype viruses, and this would in turn make establishment of secondary and systemic infections more time consuming as the virions can not utilize the more rapid, alternative route of infection (see Discussion of chapter 3), and would thus result in a slower speed of kill.

Previous reports that Ac23 is an ODV envelope protein, and my observation that Ac23-GFP fusion protein localizes to the nuclear membrane of the infected cell (a likely source of the ODV envelope), suggest that Ac23 might be interacting directly with nucleocapsid proteins to promote nucleocapsid envelopment. Whether Ac23 interacts with the major capsid protein VP39 or other proteins remains to be determined. In combination with previous reports that Ac23 is detected on the BV envelope, and may affect the production of infectious BV, these results indicate that F proteins have evolved functions beyond envelope fusion and play a different role from that of GP64 in viruses that contain both proteins. This result also support the hypothesis that packaging multiple nucleocapsids in virions and the retention of a non-essential F homolog offers selective advantage to this multicapsid nucleopolyhedrovirus.

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