Use and Control of Co-expression in the Baculovirus-Insect Cell System for the Production of Multiple Proteins and Complex Biologics

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Statement of Contributions

The work presented in this thesis has been performed either directly by me, or directed by me as the primary researcher.

The work presented in Chapter 3 is a published journal article which is co-authored with Altamash Jauhar, Jennifer Mackenzie, Sascha Kieβlich and Dr. Marc Aucoin. I am the primary author of this work.

Chapters 5 and 6 are journal articles pending submission, which are co-authored by myself and Dr. Marc Aucoin. I am the first author of both of these papers.

Abstract

The ease of use and versatility of the Baculovirus Expression Vector System (BEVS) has made it one of the most widely used platforms for recombinant protein production. In the last ten years, the system has even gained commercial acceptance for the production human biologics such as for the production of human papillomavirus vaccine (Cervarix®) and influenza vaccine (FluBlok®). The work presented in this thesis aims to further the utility of this system in coexpressing multiple proteins within a single cell, with the final goal of setting up designed and tightly controlled gene expression schemes within insect cells.

In this work we explore the effect of using different *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) promoters to control the timing and expression of multiple proteins within insect cells. We do this first in a simple two fluorescent protein system where protein and RNA expression levels of these two proteins are tracked over time and complex interaction effects are evaluated. Some of these promoter combinations are then used in a more complex and industrially relevant influenza VLP production system, in which effects such as significant post translation modification, protein trafficking to the cell membrane, insertion of protein into the membrane, and VLP budding from the surface are present. These studies coexpress two influenza proteins – the Hemagglutinin (HA) and Matrix 1 (M1) proteins genetically fused to eGFP and DsRed2 fluorescent proteins to easily track influenza protein localization and expression levels within insect cells, and to track influenza virus-like particles in culture supernatant. Lastly, we examine the effect of coexpressing superoxide dismutase as a helper protein to extend the lifespan of insect cells post-baculovirus infection.

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Contents

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Abbreviations

28S rRNA: Structural Ribosomal RNA incorporated into the 28S subunit of insect cell ribosomes

AAV: Adeno-Associated Virus

AcMNPV: *Autographa californica* multicapsid nucleopolyhedrovirus

basic: Gene encoding for the baculovirus basic p6.9 Basic protein

BEVS: Baculovirus Expression Vector System

BiP: Immunoglobulin heavy chain binding protein

cDNA: complementary DNA

chiA: Gene encoding for the baculovirus chitinase A protease

Ct: Cycle threshold value

Cu,Zn-SOD: Copper Zinc Superoxide dismutase

DAR: Downstream Activating Region

DI: Defective Interfering particles

DNA: Deoxyribonucleic Acid

DsRed2: Discosoma sp. red fluorescent protein

eGFP: enhanced Green Fluorescent Protein

FP: Few Polyhedra baculovirus phenotype

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GFP: Green Fluorescent Protein, referring to eGFP

gp64: Gene encoding for the baculovirus GP64 envelope glycoprotein

HA: Influenza Hemagglutinin

HA-GFP: Influenza Hemagglutinin genetically tagged with eGFP

HAU: Hemagglutination Units

hpi: Hours post infection

hr(s): Homologous Region(s)

IAP: Baculovirus Inhibitor of Apoptosis Protein

ie: Intermediate Early genes encoding for a class of baculovirus proteins expressed early during infection

M1: Influenza Matrix 1

M1-RFP: Influenza Matrix 1 genetically tagged with DsRed2

Mn-SOD: Manganese Superoxide dismutase

MOI: Multiplicity of Infection

mRNA: messenger RNA

NA: Influenza Neuraminidase

NPV: Nucleopolyhedrovirus

p10: Gene encoding for the baculovirus P10 protein

PAGE: Polyacrylamide gel electrophoresis

PCR: Polymerase Chain Reaction

polh: Gene encoding for the baculovirus polyhedrin protein

PTA: phosphotungstic acid

qPCR: Quantitative real time PCR

RFP: Red Fluorescent Protein, referring to DsRed2

RNA: Ribonucleic Acid

TBP: TATA-Binding Protein

TEM: Transmission Electron Microscopy

UAR: Upstream Activating Region

vcath: Gene encoding for the baculovirus v-cathepsin protease

VLF-1: Very Late Factor-1

Chapter 1 : Introduction

The baculovirus expression vector system (BEVS) has grown to be one of the most widely used protein production systems. The system uses one or more baculoviruses to introduce one or more genes of interest into insect cells where the recombinant protein(s) of interest is (are) expressed. The popularity of the system stems from factors such as the ability to grow insect cells in high density suspension cultures, the mammalian-like post translational modification capability of insect cells, the capability of the baculovirus genome to accept multiple large insertions, and the high protein yields obtained using the system. In addition, the limited host range in which these viruses can replicate provides a very high level of biosafety for the production of therapeutics for human use, as compared to mammalian cell culture.

While superior in many ways as a protein production system to mammalian cell culture, there are some limitation of the BEVS. In its most widespread implementation, the proteins of interest are produced only in the very-late phase of the baculovirus infection cycle through the use of two very strong promoters: the *polh* and *p10* promoters. Though these promoters drive the production of very large amounts of protein, they are active at a time when the cell is dying and cellular transcription and translation has largely shut down, and as a consequence, cellular post-translation and secretory mechanisms are perturbed (Jarvis and Summers, 1989). In addition, the presence of viral and cellular proteases can cause the degradation of susceptible protein products (Ikonomou et al., 2003). Much work has been done to circumvent these problems such as producing proteins earlier during the infection cycle and the introduction of protease inhibitors (Ikonomou et al., 2003), though these methods are not commonly used in practice.

The BEVS is especially suited to the simultaneous expression of multiple proteins within a single insect cell, due to factors such as the large capacity for insertion into the baculovirus genome and ability to manipulate expression levels by controlling the amount of baculovirus added. This is useful in the production of multi-subunit proteins, as well as in the co-production of helper proteins such as chaperones, which can help in increasing the production of recombinant proteins of interest. While much work has been done in this respect, the simultaneous production of multiple proteins can still be optimized in several ways. The theme of the work presented in this thesis deals with controlling co-expression of multiple proteins within insect cells.

The driving hypothesis behind this work is that promoter control can be used to control the timing and level of protein expression in an insect cell coexpression system. In addition, it was hypothesized that the simultaneous high level production of two proteins in an insect cell can result in the protein coding genes "competing" for cellular resources; a reduction of this competition effect can be achieved by temporally separating the expression of these genes. This control can be achieved using promoters to control levels of protein expression within a cell. We also aimed to better understand the dynamics involved in the production of multiple foreign proteins within insect cells in the BEVS system.

The thesis starts with this general introduction to the work, followed by a review of relevant literature presented in Chapter 2. Following this, the various projects are presented in the form of individual chapters.

The first project was a collaborative effort with Altamash Jauhar, who was a Masters of Applied Sciences student in the lab of Dr. Aucoin. In this, a simple system was first implemented where two fluorescent proteins, a green fluorescing eGFP and a red fluorescing DsRed2, were

co-expressed within a single cell. These proteins were expressed under different promoter combinations, and the resulting fluorescence was closely monitored by flow cytometry. This study revealed that different promoter combinations resulted in extremely well defined ratios of the two fluorescent proteins being expressed over time. In addition, this work demonstrated that clear competition effects are present when producing multiple proteins simultaneously within insect cells. This study was especially important as it allowed for the examination of the protein production process of two very simple proteins that did not have to undergo extensive post-translational modifications or cellular trafficking. The results of this study have been published in a journal article in the journal Biotechnology and Bioengineering, and this is presented in Chapter 3 of this thesis. This study achieved the goals set out in the hypothesis statement in a simple system.

One limitation of the first study was that it was not clear if the phenomenon of competition occurred at the transcription or translation steps. In order to resolve this, a second study conducted with Altamash Jauhar examined the ratios eGFP and DsRed2 RNA transcripts being produced over time from the different constructs co-expressing the two fluorescent proteins. This was done by semi-quantitative Real Time PCR. While this study suffered from a lot of noise in the data, some trends could be inferred, indicating that competition was present at the RNA transcription phase. The results of this study are presented in Chapter 4.

The third study sought to examine these effects in a more complex and relevant protein system. To this end, the production of influenza VLPs by the co-expression of influenza hemagglutinin (HA) and matrix 1 (M1) proteins in insect cells was examined. The examination of previous studies such as that by Thompson et al (2015) convinced us that a modified system was essential to reduce the amount of effort needed to reliably detect the production of influenza

proteins within insect cells and to examine their incorporation into VLPs. This was done by genetically modifying the HA and M1 genes to incorporate eGFP and DsRed2 genes, respectively, into sites that have been shown to be amenable to insertions (such that the functions of the HA and M1 proteins are minimally affected). The expression of these modified proteins within insect cells allowed for the clear visualization of protein production within cells by microscopy and its quantification by flow cytometry. In addition, the produced virus-like particles could be detected via flow cytometry due to their fluorescence. The work on production of fluorescent influenza proteins and their incorporation into VLPs is detailed in an article submitted to the Journal of Biotechnology and presented in Chapter 6. The subsequent manipulation of influenza protein expression levels is described in Chapter 7. This study was similar to that conducted in Chapter 3 in that it established the utility of promoter combinations to control the timing and level of production of coexpressed proteins in insect cells, albeit in a more complex system. It also addressed the stated objective of the work, to examine effects of competition in coexpressing complex proteins within insect cells.

The fourth and final study details the use of co-expression in BEVS to attempt to extend the lifespan of baculovirus infected insect cells through the co-expression of a copper-zinc superoxide dismutase (Cu/Zn SOD), along with a gene of interest (in this case RFP). It was thought expression of superoxide dismutase could relieve oxidative stress on the cell and help maintain the viability of the culture for a longer period of time. This study, presented in Chapter 8, was conducted with the help of an NSERC URA student Jenna Collier and showed that the expression of Cu/Zn SOD did not induce protective effects in infected Sf9 insect cells.

Chapter 2 : Literature Review

2.1. Baculoviruses

Baculoviruses are enveloped DNA viruses that have very narrow host specificity within arthropod invertebrates (Miller & Lu, 1997). They can be divided into the nucleopolyhedroviruses (NPVs), which can form occlusion bodies composed of one or more virions encapsulated in a polyhedrin protein matrix, and the granuloviruses (GV), which can form occlusion bodies containing a single virion encased in a granulin protein matrix (Funk et al., 1997). Baculoviruses can exist in one of two states during their life cycle: the occluded form, which are responsible for transmission between hosts, or the budded form, which are responsible for transmission within a host. Baculoviruses are large viruses nearly 300 nm in length and 50 nm in diameter, with a circular supercoiled double stranded DNA genome that is between 80 – 180 kb in size encoding between 90 – 180 genes (Rohrmann, 2011). The *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) was one of the first to be extensively studied and used for purposes such as biological pest control and for protein production.

2.2. The baculovirus replication cycle and gene expression

Within insect cell culture, baculovirus transmission is mainly mediated by absorptive endocytosis (Blissard and Wenz, 1992; Volkman and Goldsmith, 1985), with subsequent release of the nucleocapsid into the cytoplasm by fusion of the baculovirus membrane to the endosome membrane. The latter is mediated by the gp64 baculovirus surface glycoprotein (Blissard and Wenz, 1992). The nucleocapsids then migrate to nuclear pores and seem to enter the nucleus through these pores (Ohkawa et al., 2010). The viral genome is believed to be released by

destabilization of the capsid caused by phosphorylation of the core protein p6.9 (Funk and Consigli, 1993). The released viral genome is then used for viral RNA synthesis and multiplication of viral DNA. An overview of the baculovirus life cycle is presented in Figure 2-1

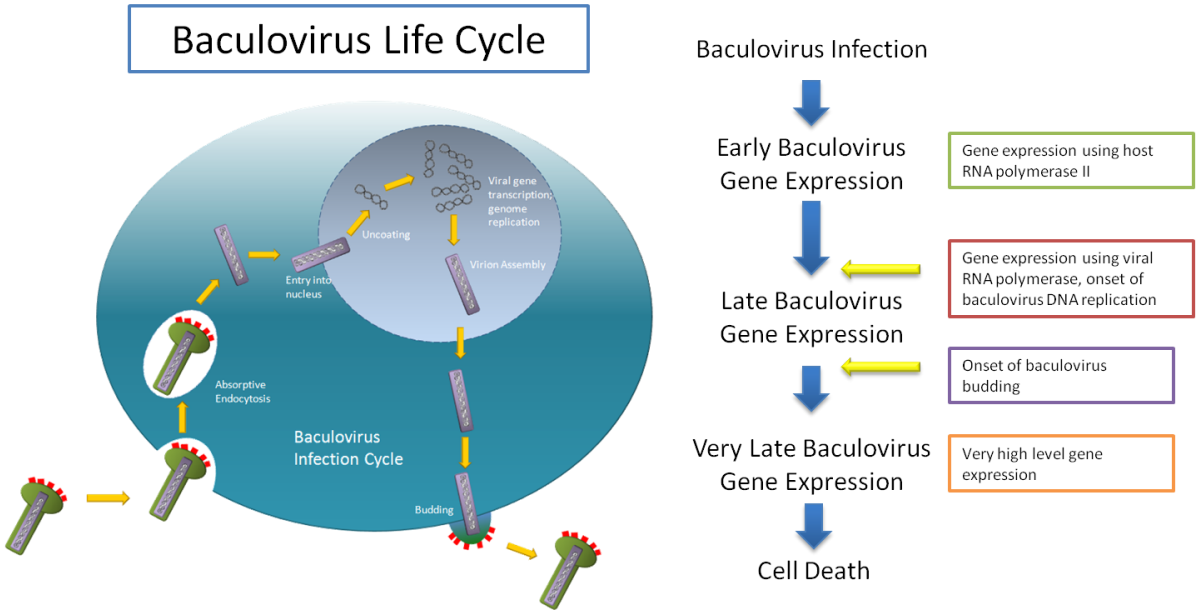

Figure 2-1: Baculovirus life cycle and gene expression

The baculovirus infection cycle is temporally divided into three phases: the early phase, the late phase, and the very-late phase of infection. The expression of baculovirus early phase genes occurs from about 30 minutes (Chisholm and Henner, 1988) to about 6 hours post infection (Kelly et al., 2007) and is divided into two sub-phases, with the delayed early phase genes requiring the expression of immediate early genes. Genes in this phase are transcribed using host RNA polymerase II (Grula et al., 1981) and largely code for transcriptional activators and enhancers. These include genes coding for products like the Immediate Early trans-regulators (IE0, IE1 and IE2). Of these, IE1 is one of the most important and is involved in early gene regulation and DNA replication. The onset of baculovirus DNA replication occurs at the same time as the beginning of late gene transcription (Rohrmann, 2011) and is discussed later in

this review. The onset of baculovirus budding occurs at the end of baculovirus DNA replication, which corresponds to the end of the late phase of the infectious cycle (Rohrmann, 2011). The transcription of late and very-late phase genes is associated with the activity of a virally encoded multisubunit RNA polymerase (Fuchs et al., 1983; Guarino et al., 1998). The transcription of late phase genes begins at about 6 hours post infection and continues till the expression of very-late phase genes at about 20 hours post infection (Kelly et al., 2007). The *polyhedrin* and *p10* genes are two genes that are expressed at very high levels during the very-late phase of infection (Smith et al., 1983b) and are involved in the formation of occlusion bodies.

The origin of baculovirus DNA replication is not clearly known, with some reports indicating that the homologous regions (hrs) are the sites of DNA replication initiation (Kool et al., 1993), with others suggesting that initiation can occur at other regions (Habib and Hasnain, 2000; Lee and Krell, 1992). The replication is thought to occur either by a rolling circle mechanism (Oppenheimer & Volkman, 1997) or by recombination (Okano et al., 2007) and requires the action of several baculovirus gene products. In addition to the DNA polymerase and helicase proteins encoded by the baculovirus *dnapol* and *helicase* (*p143*) genes, the gene products of the *ie1*, *late expression factor (lef)-1*, *lef-2*, *lef-3*, *lef-7*, and *ie-n* genes are necessary for efficient baculovirus DNA replication (A. Lu & Miller, 1995). It has also been suggested that host proteins also play a role in this process (Rohrmann, 2011).

Baculovirus infection of insect cells causes the arrest of the cell cycle at the G2/M phase (Braunagel et al., 1998). In addition, it also causes a decrease in host transcription during the late phase of infection (Nobiron et al., 2003) and an almost universal shut-off of host protein translation from about 6 hours post-infection (Carstens et al., 1979). Another important characteristic of baculovirus infection of insect cells is the production of anti-apoptotic factors,

which counteract the natural infection-induced apoptosis response. The *p35* gene product is produced during the early and late phases of baculovirus infection and blocks the apoptotic response, in addition to having a role in baculovirus gene expression (Hershberger et al., 1994). The *p35* gene product is also capable of inhibiting apoptosis in a wide range of cell types (Clem, 1997). Another class of genes called the inhibitor of apoptosis (*iap*) genes, common in viruses and eukaryotes, also exist in baculoviruses (Rohrmann, 2011). It is thought that both P35 and IAP inhibitors function by blocking the activity of caspases in infected insect cells (Bump et al., 1995; Rohrmann, 2011). The baculovirus replication cycle and its molecular biology have been extensively reviewed elsewhere (Miller, 1997; Rohrmann, 2011)

2.3. Control of baculovirus gene expression

Baculovirus early promoters are transcribed by the host RNA polymerase II and so, the control elements found in early promoter expression are similar to those of insect cells. It has been shown that the TATA box element, which is a common site for assembly of the RNA polymerase pre-initiation complex in eukaryotes (Karp, 1996), is important for the activity of most baculovirus early promoters like that of the *gp64* gene (Blissard and Rohrmann, 1991; Blissard et al., 1992) and *ie1* (Pullen and Friesen, 1995a) early promoters. This activity is through the binding of the TATA-binding protein (TBP), which allows for the assembly of the RNA polymerase II complex (Karp, 1996). An additional control fragment is the CAGT consensus fragment, which is thought to be involved in positioning the RNA start site (Rohrmann, 2011) and can also function as activators of transcription, with many promoters containing a TATA box also containing a CAGT motif (Friesen, 1997; Rohrmann, 2011). In addition to these, and other less common elements, downstream activating regions (DARs) such as (A/T)CACNG present in the 5’ untranslated region of transcripts, can also be important for the

activity of some promoters such as the *ie1* (Pullen and Friesen, 1995b) and *gp64* (Kogan et al., 1995) promoters, and may work to stabilize the RNA polymerase complex interaction with the upstream CAGT sequence (Friesen, 1997). Several early baculovirus promoters also use upstream activating regions (UARs) such as GATA and CACGTG (Kogan et al., 1995) which are located before the TATA box motif and that work by binding host transcription factors, which in turn activate downstream early promoters as in the case of promoters such as the *gp64* (Kogan and Blissard, 1994) and pe38 (Krappa et al., 1992) promoters. These UARs may be found significantly upstream of the promoter as in the case of the *ie1* promoter where the UARs can be found several hundred base pairs in front of the transcriptional start site (Pullen and Friesen, 1995b). The activity of early promoters can also be greatly enhanced by their proximity to baculovirus homologous regions or *hrs*, eight of which are found in the baculovirus genome (Ayres et al., 1994). They consist of 60 – 70 bp repeats containing imperfect 28bp palindromes. These have been shown to increase transcription of cis-linked early promoters (Guarino et al., 1986; Nissen and Friesen, 1989), probably by recruiting host transcription factors. Their activity seems to be limited yearly promoter recruiting of RNA polymerase II (Rodems and Friesen, 1993), which is drastically increased by the action of the baculovirus IE1 trans-activating protein (Guarino et al., 1986; Nissen and Friesen, 1989; Rodems and Friesen, 1995) which either binds to the hr 28bp palindromes as a dimer (Rodems and Friesen, 1995), or stabilizes host factor interactions with the hr region (Friesen, 1997). This interaction works to increase transcription of cis-linked genes either by direct interaction with the polymerase complex, or by altering DNA or chromatin conformation, which could increase transcription from the linked promoter (Friesen, 1997).

IE1can also transactivate genes in the absence of hr regions, though at much reduced levels, and there is some preliminary evidence suggesting that this non-hr dependent mechanism may require the presence of host factors (Murges et al., 1997). Other important transactivators of early gene expression include the immediate early regulators IE0, IE2 and PE38. Comprehensive reviews of baculovirus early gene expression have been published elsewhere (Friesen, 1997; Rohrmann, 2011).

Baculovirus late and very-late gene expression occurs after the onset of DNA replication and requires the presence of a baculovirus RNA polymerase (Fuchs et al., 1983; Guarino et al., 1998). This polymerase is one of the simplest eukaryotic DNA-dependent RNA polymerases known and consists of two copies of the baculovirus encoded polypeptides p47, LEF-4, LEF-8 and LEF-9 (Guarino et al., 1998). The most important element present in late and very-late promoters is the (A/G/T)TAAG motif (Lu and Miller, 1997; Rohrmann, 2011). This element, along with the ~12 - 18bp flanking regions were found to drive downstream protein expression at nearly wild-type levels for the gp64 promoter (Garrity et al., 1997) and to a somewhat reduced extent for the vp39 promoter (Morris and Miller, 1994). The mechanism of action of this motif is unclear but has been speculated to involve binding of transcription factors (Morris and Miller, 1994). In addition, promoters which are active during both the early and late phases of infection, such as the gp64 promoter, contain a hybrid TATAAG element, which may act as an activator for both early and late transcription (Blissard and Rohrmann, 1991; Rohrmann, 2011). Very-late promoters are also controlled by the TAAG sequence, but differ in that the TAAG motif and short flanking regions are not sufficient for high level promoter activity (Morris & Miller, 1994), which indicates that the regions surrounding the TAAG motif have a significant impact on promoter activity, possibly by modulating the binding of different transcription factors (Morris

and Miller, 1994). It has been found that 18 baculovirus genes, including various LEFs, the proteins in the DNA polymerase complex, and IE1 and IE2 are necessary for optimal expression from several late and very-late promoters (Lu and Miller, 1997). An additional regulator element in very-late promoters is the "burst sequence" which is present between the TAAG motif and the translation initiation codon and which is needed for the very high levels of expression at the very-late phase of the infectious cycle (Ooi et al., 1989). There is evidence that this sequence activates transcription by binding to the very late factor 1 (VLF-1) protein (Mistretta and Guarino, 2005; Yang and Miller, 1999), which only activates very-late baculovirus genes. The activity of late promoters is not increased by the inclusion of the *polh* burst sequence downstream of late promoters (Morris and Miller, 1994) or by the activity of VLF-1 (Todd et al., 1996). This indicates that additional sequences, found only in baculovirus late promoters are necessary to recruit VLF-1 or other factors, to the burst sequence. In addition, *polh* promoter activity is modulated by factors in insect cells, which may vary between different cell lines, and even between individual cells within a culture (Cheng et al, 2013). An excellent review of baculovirus gene expression in the late and very late phases of infection has been published elsewhere (Lu and Miller, 1997).

There is not much data regarding translational control of baculovirus mRNAs, with several late genes containing mini-cistrons, which may serve to regulate gene expression (Chang and Blissard, 1997). It is assumed that the other aspects of translation of mRNA to protein in baculovirus infected insect cells are similar to those found generally in eukaryotes (Karp, 1996; Lu and Miller, 1997).

2.4. The utility of baculovirus and BEVS

The narrow host range and high biosafety of baculoviruses has resulted in them being used in a wide range of practical applications such as highly selective insecticides, as gene therapy vectors, for screening applications using baculovirus surface display and, most importantly, as vectors for protein production in insect and mammalian cells, and in insect larvae.

The BEV system has become one of the most widely used systems for protein production (Kost et al., 2005). This stems from its large protein production capacity, which can be compared to prokaryotic systems (Jarvis, 1997), and the inherent biosafety and eukaryotic protein processing capabilities of insect cells. In addition, the ability of multiple baculoviruses to infect the same cell, which coupled the large gene packaging capacity of the baculovirus, makes this system well suited to the production of multiprotein complexes. In addition, the ability of insect cells to be grown in suspension culture and their high tolerance to osmolarity and by-product concentrations (Ikonomou et al., 2003) allow for easier maintenance of these cultures, as compared to mammalian cells.

The BEV system makes use of baculoviruses as gene delivery vehicles into permissible insect cell lines or larvae (Kost et al., 2005). This is done by inserting one or more genes of interest, using transfer plasmids, into the baculovirus genome under the control of a baculovirus or insect cell promoter and at a site that will not interfere with baculovirus replication. These have traditionally been sites coding for non-essential genes such as the *polh* gene (Smith et al., 1983a), and more recently, the *chiA* and *vcath* protease genes (Fitzgerald et al., 2006). Furthermore, the gene of interest is most often placed under the control of the very strong baculovirus *polh* or *p10* promoters (Smith et al., 1983b), which results in very high expression levels of the gene of interest. However, the use of other promoters to express genes at earlier

times during the infection cycle has been explored, and is discussed in greater detail in section 2.8.

2.5. Limitations of BEVS

The BEVS is limited in its protein production capability in several ways: the lack of completely mammalian-like post translational modifications, the breakdown of secretory pathways in the very-late phase of the infectious cycle, the high amount of proteolysis in the expression system, and issues with baculovirus vector instability. While these issues may not be significant for all proteins, it does reduce the general utility of this system. In addition, the BEVS is a transient system requiring the use of baculoviruses, a process which is more complicated than using continuously producing cell lines. A further problem with this system is the presence of baculovirus in the supernatant at the end of a product run, which can complicate product recovery.

2.5.1 Post-translational modifications

While BEVS is capable of producing proteins with eukaryotic post-translational modifications such as glycosylation and proteolytic processing such as the cleavage of signal peptides, the pathways and end-products are not identical to those obtained from cell lines from higher organisms. A further complicating factor is the shutdown of host protein synthesis pathways, which can further affect the post-translational modification capability of the host. The problem is exacerbated by the large amount of protein produced by traditional *polh* promoter vectors, where high expression can overwhelm the host cell's weakened protein modification machinery.

2.5.1.1 Glycosylation

Glycosylation is an important factor for the biological activity of many human therapeutic proteins, and is therefore an important consideration in choosing a cell culture platform for therapeutic production. Glycosylation in insect cells differs from that of mammalian cells in the sugar structure pattern appended to the proteins. In particular, insect cells elongate trimmed N-glycan precursors with mannose residues to produce high-mannose structures (Jarvis and Finn, 1995; Kulakosky et al., 1998), though this may be product specific and not a general trend (Davidson et al., 1990; Davidson and Castellino, 1991). Insect cell lines such as Ea4 (Ogonah et al., 1996) and DpNI (Palomares et al., 2003) have been shown to be capable of more complex glycosylation than cell lines such as Sf9. Modified glycosylation patterns in insect cells have been obtained by the introduction of glycosyltransferases such as galactosyltransferases and sialyltransferases. A good example of this strategy is the generation of a cell line incorporating five glycosyltransferases, as well as two enzymes involved in sialic acid synthesis. This allowed for the efficient sialylation and mammalian-like glycosylation of glutathione-S-transferase (Aumiller et al., 2003). In addition, the production of glycoproteins earlier during the infectious cycle has been explored to avoid the reduction in post-translational modifications during the very-late phase of infection.

2.5.1.2 Secretion

The secretory pathway in insect cells is significantly perturbed during the very-late phase of infection and can be overwhelmed by large amounts of recombinant protein produced during this phase. As a result, secreted and membrane bound proteins of interest are often produced in an insoluble form (Ailor and Betenbaugh, 1999). One strategy to overcome this limitation involves the expression of chaperones such as heat shock protein 70 (Hsp70) (Ailor and Betenbaugh,

1998), calreticulin (Kato et al., 2005) and binding immunoglobin protein (BiP) (Hsu and Betenbaugh, 1997), which have been shown to increase solubility and secretion of proteins of interest in insect cells. The use of chaperones to improve foreign protein production has been reviewed extensively elsewhere (Ailor and Betenbaugh, 1999; Sokolenko et al., 2012). Production of these secreted and membrane bound proteins at earlier times during the infection cycle, using earlier promoters, has also been studied to improve correct localization of the proteins of interest (Grabherr et al., 1997; Higgins et al., 2003; Lawrie et al., 1995). In addition, cell lines such as the *Trichoplusia ni* derived cell lines BTI-TN-5B1-4 High Five (Hi5) and *BTI-Tnao*38 cells allow for much greater secretion of proteins out of the cells (Wilde et al., 2014).

2.5.2 Proteolysis

The production of cysteine proteases (Gotoh et al., 2001) during the baculovirus infection cycle can affect the quality and quantity of recombinant protein when using the BEVS. These proteases are produced both by the cell, as a stress response or during cell lysis, and by the baculovirus, to facilitate baculovirus release from the cell. Baculovirus proteases such as the v-cath protease from the *vcath* gene (Slack et al., 1995b), or chitinase from the *chiA* gene (Hawtin et al., 1995) are produced, along with cellular proteases, during the very-late phase of the baculovirus infection cycle. Strategies have been developed to counter the issue of proteases including deleting the baculovirus *vcath* and *chiA* protease genes (Fitzgerald et al., 2006; Monsma and Scott, 1997), and the use of protease inhibitor cocktails (Gotoh et al., 2001), which increase product yield and stability. Proteolysis in the baculovirus system and methods to reduce its effect on protein production has been reviewed elsewhere (Gotoh et al., 2002; Ikonomou et al., 2003).

2.5.3 Baculovirus vector instability

One of the issues with the BEVS is baculovirus instability, where the gene expression cassette containing the gene(s) of interest is deleted (Carstens, 1982). Defective interfering (DI) viruses with up to 43% of their genome deleted can accumulate upon repeated passaging of baculovirus in cell culture (Kool et al., 1991; Pijlman et al., 2001). This genome deletion is accompanied by the enrichment of a non-essential non-hr origin of replication (ori) within the baculovirus genome (Kool et al., 1993; Lee and Krell, 1992). Recent research has shown that deletions within the non-hr ori region can reduce these gene deletion events (Pijlman et al., 2003); however, this stability does not extend to the retention of inserted foreign gene sequences into the genome. Stable foreign gene integration is dependent on the site of insertion of foreign genes. The formation of DI viruses may require an intermediate step where transposon sites accumulate in some regions of the baculovirus genome (Carstens, 1987). Formation of DI viruses can be delayed by the modification of some of these sites within the *fp25k* gene (Giri et al., 2012). Interestingly, the accumulation of mutations within the *fp25k* gene is also thought to be responsible for the few polyhedra (FP) phenotype of baculovirus (Jarvis et al., 1992; Cheng et al., 2013; Harrison et al., 1996). The integration of a hr1 region in the opposite orientation into the foreign gene expression cassette in the Bac-to-Bac system, improves baculovirus vector stability, as well as foreign gene expression (Pijlman et al., 2003). To improve the BEVS, Vijayachandran et al. (2012) has set out to create a rationally designed minimal genome baculovirus to remove sources of baculovirus genome instability and generate a small stable baculovirus vector for large scale protein production.

2.6. Multiple protein production within a single cell

The ability of the BEVS to enable the expression of multiple foreign genes concurrently makes this system well suited to the production of complex proteins such as antibodies (Song et al., 2010; zu Putlitz et al., 1990) and virus-like particles (Betenbaugh et al., 1995; Crawford et al., 1994; French and Roy, 1990). In addition, the production of some proteins may be enhanced by helper functions of other proteins, such as chaperones, which can be co-expressed in the insect cell system (Ailor and Betenbaugh, 1998; Hsu and Betenbaugh, 1997; Kato et al., 2005). The expression of multiple foreign genes in a single cell in the BEVS can be done either: by infecting a culture with multiple viruses, each carrying one foreign gene (co-infection); by using a single virus carrying multiple foreign genes (co-expression); or a combination of the two. Each system has unique advantages and disadvantages. The use of a co-infection system allows for easy manipulation of expression levels of various genes by controlling the multiplicity of infection (MOI) as well as by controlling time of infection (TOI) of different viruses, by which temporal separation of the expression of various genes can be achieved. However, the production of complex proteins requires that each cell be infected with at least one of each type of virus. This is complicated by the fact that virus infection in cell culture is known to behave as a Poisson process (Licari and Bailey, 1992), with some cells being infected by multiple viruses, some with only one, and some cells not being infected at all. This is especially disadvantageous in multi-subunit protein production with multiple viruses, where the infection of a cell with one virus and not the other leads to a non-productive infection event from the perspective of the assembly of the complex protein of interest.

The use of a polycistronic virus encoding for multiple genes is made possible by the large capacity of the baculovirus genome for accepting foreign gene insertions. The use of one virus to

introduce all necessary genes removes the problems associated with the co-infection process. The benefits of co-expression over co-infection have been shown repeatedly, with the use of polycistronic vectors consistently producing higher yields of final product (Pushko et al., 2005; Roldao et al., 2006; Shanks and Lomonossoff, 2000; Vieira et al., 2005). In addition, the introduction of multiple genes using a single vector reduces the overall MOI in the culture, which could reduce stress on the infected cell and could lead to increased cell longevity (Roldao et al., 2006). However, the manipulation of gene ratios and timing of expression can only be conducted by the manipulation of promoters and other regulatory elements driving the expression of the different genes, and this makes co-expression unsuitable for exploratory purposes. Therefore, co-infection is suitable when determining the levels of individual genes needed to make a suitable product efficiently, while co-expression is more suited for when this exploratory work is completed and large scale production is desired. However, there is a dearth of information regarding the strength and expression profiles of promoters active in insect cells, which complicates the task promoter selection for co-expression.

Tailoring the expression levels of different component proteins produced in the BEVS system can be important for several reasons. The first is to modulate levels of component proteins in multi-subunit proteins, as has been demonstrated in the production of VLPs. Parvoviruses are one such example, where the levels of component proteins can affect the composition of the final product (Tsao et al., 1996). However, even in cases where VLP compositions are unaffected by the expression levels of component proteins, the proteins that are expressed over-abundantly are wasted, leading to unnecessary depletion of cellular resources (Sokolenko et al., 2012). Tailoring expression levels is also important in cases where levels of helper genes need to be carefully managed to be helpful to the production of the protein of

interest. One of the earliest references to this idea was to tailor helper chaperone production in a dual protein expression BEVS (Ailor and Betenbaugh, 1998).

There have been several vector designs that allow the construction of baculovirus that carry multiple genes. These include commercial vectors such as pOET5 from Oxford Expression Technologies™, which uses homologous recombination to insert foreign genes into the *flash*bac™ family of baculovirus genomes. It should be noted that the *flash*bac™ family of baculovirus genomes contain several deletions of proteases and non-essential genes. A similar system is the pFastBac™ Dual vector from Thermo Fisher Scientific Inc, which uses a transposition based Bac-to-Bac® system to generate recombinant baculoviruses. A further extension of these systems is the MultiBac™ system from Geneva Biotech, where multiple genes can be introduced to two sites within a baculovirus genome with the *chiA* and *vcath* protease genes deleted, through Tn7 transposition and Cre-LoxP recombination (Fitzgerald et al., 2006; Fitzgerald et al., 2007b). This has used to express up to 6 genes from a single baculovirus (Fitzgerald et al., 2006). More recently, genes for up to seven proteins have been added in a single baculovirus vector (Kanai et al., 2013) by inserting each gene into different loci of the genome. There is still greater potential for the vector as 8 sites, other than the *polh* locus, are amenable to insertion of foreign sequences (Noad et al., 2009).

2.7. Competition among co-expressed genes

There is evidence in the literature pointing to competition effects when expressing multiple proteins in the BEVS. The deletion of genes downstream of the very strong very-late promoter p10 has been linked to improved transcription (Chaabihi et al., 1993) and translation (Hitchman et al., 2010) of genes driven by the polh promoter. It has been speculated that this may be due to limitation in supply of host or virally encoded transcription factors necessary for the activity of

the *polh* promoter, and for which the *p10* promoter is able to compete for more successfully (Lu and Miller, 1997). Although the expression of the chaperone calnexin causes an increase in production of a protein of interest (Kato et al., 2005; Tate et al., 1999); the expression of calnexin along with another chaperone such as calreticulin or immunoglobulin heavy chain binding protein (BiP) causes a decrease in expression of the protein of interest. The latter has been speculated to be due to overloaded cellular protein synthesis machinery caused by the simultaneous high level expression of three genes under the control of the strong *polh* promoter (Kato et al., 2005). Further evidence comes from research in a system producing AAV virus using three baculoviruses (BacCap, producing the AAV capsid proteins; BacRep, producing AAV replication proteins necessary for AAV genome replication and encapsidation; and BacITRGFP, which supplies the AAV genome for packaging), in which increasing the expression of proteins from the BacRep virus (by increasing the MOI) causes a decrease in the number of non-genome containing virus particles (coded for by the BacCap virus). This has been speculated to be due to the effect of competition between the production of replication proteins and capsid proteins (Aucoin et al., 2006a).

2.8. Use of alternate promoters in BEVS

The vast majority of proteins produced in the BEVS system have been produced using baculovirus very-late *polh* or *p10* promoters to drive foreign gene expression. While these promoters are some of the strongest known natural promoters, they are active only during the very-late phase of the baculovirus replication cycle in an insect cell, when the post-translational and secretory mechanisms of the cell are severely perturbed, and when high levels of cellular and baculovirus proteases are present in the system, as discussed earlier. The situation is further complicated by the fact that the cellular machinery may be overwhelmed by the large amount of

recombinant protein being produced under the control of the very-late promoters. Therefore, several groups have sought to investigate the use of promoters that are active earlier during the baculovirus infection cycle even though they would not be as strong as the *polh* and *p10* promoters. Conversely, promoters stronger than the *polh* promoter have been produced through mutations within the promoter region (Rankin et al., 1998; Ooi et al, 1989) or by screening naturally occurring promoters from non-AcMNPV baculoviruses (Martínez-Solís et al, 2016).

When expressing complex eukaryotic protein requiring post-translational modifications, the baculovirus *ie1* promoter, though weaker, has been shown to allow as much or even more active protein to be produced, as compared to the *polh* promoter (Jarvis et al., 1996). Other promoters such as the *basic* protein promoter (Bonning et al., 1994; Chazenbalk and Rapoport, 1995; Higgins et al., 2003) and the large *gp64* promoter (Grabherr et al., 1997) have also been shown to also produce as much or greater amounts of complex proteins- proteins requiring secretion or trafficking to the cell surface, and multi-subunit proteins (Higgins et al., 2003). In nature, these promoters drive the production of the baculovirus p6.9 Basic DNA binding protein and the surface expressed baculovirus GP64 glycoprotein, respectively. Further control over expression levels and times have been achieved through modified promoters such as: a *Pcappolh*, a hybrid of the *vp39* capsid and *polh* promoters (Thiem and Miller, 1990); tandem *ie1* promoters (Kojima et al., 2001); synthetic early promoters (Blissard et al., 1992); constitutive insect promoters like the *hsp70* promoter (Lu et al., 1996; Prikhod'ko et al., 1998); and truncated *p10* and *ie1* promoters (Urabe et al., 2002; Urabe et al., 2006). A further possibility for modulating expression levels comes from addition of enhancer elements such as the baculovirus homologous regions (Guarino et al., 1986). Addition of the *hr3* region ahead of a late *vp39*

promoter has been shown to induce onset of transcription from this promoter 10 hours before the unmodified *vp39* promoter (Ishiyama and Ikeda, 2010).

Promoters having earlier activity have shown benefit for the production of a two component simian immunodeficiency virus (SIV) VLP consisting of Env and Gag proteins. Driving the expression of the membrane associated Env protein using a hybrid late/very-late promoter resulted in a greater expression of Env on the cell surface, as opposed to Env produced using a very-late promoter (Yamshchikov et al., 1995). In addition, early promoters have been used to introduce non-native glycosylation abilities, which allow insect cells to replicate glycosylation patterns found in higher eukaryotes (Jarvis and Finn, 1996).

2.9. Influenza A VLP production

2.9.1. Influenza A, and VLPs as influenza vaccines

Influenza A viruses are enveloped RNA viruses belonging to the Orthomyxoviridae family of viruses that are a significant cause of morbidity and mortality in the modern world. Laboratory strains are usually spherical ranging from 80 – 120 nm in diameter (Lamb and Choppin, 1983). The genome of these viruses are divided into 8 negative sense, single stranded RNA fragments, five of which encode a single protein each while the other three code for two proteins each (Bouvier and Palese, 2011). Of these, the hemagglutinin (HA) gene encodes for the HA glycoprotein which is found on the surface of the influenza A virus in its trimeric form. This protein mediates the binding of the influenza virus to sialic acid residues on target cells, and assists in membrane fusion between virus and cell (Wiley and Skehel, 1987). The release of newly formed virus from cell surfaces requires influenza neuraminidase (NA) surface glycoprotein, which cleaves the sialic acids binding the cell to the virus HA protein trimer. The third important structural component of the influenza virus is the matrix M1 protein which binds

viral RNA and, along with HA and NA, make up the structural component of the virus. Other components such as the M2 protein and various non-structural proteins are necessary for virus infectivity and transmission of host cells.

The virus hemagglutinin (HA) protein is a single pass type I membrane protein that is often localized to the host apical membrane in epithelial cells. The first 17 amino acid residues correspond to a nuclear localization signal. Following this is a 511 aa extracellular domain, followed by a helical 21 aa transmembrane domain, which is followed by a 16 aa cytoplasmic tail domain (Winter et al., 1981). The M1 protein is a 252 aa protein that is arranged in a series of helical domains, with the N and C terminals facing towards the inside of the influenza virion (Shishkov et al., 1999).

While vaccines are effective in preventing or reducing the severity of the disease, the high rate of mutation and genetic re-assortment in these viruses requires the production of new vaccines every flu season. The two most common types of influenza vaccines currently used contain either live attenuated influenza virus or inactivated virions, and both are currently produced using embryonated chicken eggs. This is, however, a time and resource intensive process and is unsuitable for the production of vaccines against recent H5N1 viruses, which kill the chicken eggs (Quan et al., 2007). In addition, an influenza pandemic may significantly affect the supply of eggs for vaccine production. Therefore, it would be advantageous to develop a cell culture based system for the rapid production of influenza vaccines that would allow for quick responses to changes in influenza subtypes circulating in the human population, and against pandemic influenza. In addition, cell based vaccine production methods ensure that vaccines against dangerous influenza subtypes can be generated without any concerns of releasing those genes into the environment (Latham and Galarza, 2001). Protein Sciences has been a leader in

this field with their insect cell produced Flublok® anti-influenza vaccine, which has been increasingly used for routine vaccination, especially for patients who have egg allergies.

VLPs, which are incomplete and non-replicative virus-like particles, have been explored as potential vaccine candidates for several viruses (Pattenden et al., 2005). VLPs are professionally presented by antigen presenting cells (APCs) which in turn activate the adaptive immune system (Buonaguro et al., 2006; Kang et al., 2009; Sailaja et al., 2007). The advantage of VLPs over soluble immunogenic antigens lies in the potential of VLPs to present the antigen in a similar context to the antigen on an infective virus particle (Kang et al., 2009). VLPs containing influenza hemagglutinin (HA) have been able to induce much higher protective serum antibody responses against highly pathogenic H5N1 influenza in mice, when compared with antibody responses elicited by recombinant HA protein (Bright et al., 2007; Mahmood et al., 2008). VLPs also cause the production of antibodies that have a broader cross reactivity with antigenically different strains of the subtype of virus on which the VLPs were based (Bright et al., 2007). However, heat treated influenza VLPs were found to induce a lower titer of intact influenza specific antigens, and provide almost no protection against influenza challenge, due to the denaturation of the HA surface protein (Quan et al., 2007), thereby establishing the importance of the HA antigen in VLP based vaccines. There is also some evidence indicating that influenzaVLPs can induce a cytotoxic T cell response in mice, which may play an important role In protection from heterotypic subtypes of the influenza virus(Hemann et al, 2013).

The work presented in this thesis deals with VLPs formed from the co-expression of HA and M1.

Within influenza virus infected cells, the trafficking of HA occurs with its synthesis and localization within the endoplasmic reticulum. Following this, it is transported to the golgi apparatus, where it interacts with the M1 protein. After this, the HA is transported to lipid rafts on the cell surface (Takeda et al., 2003). Targeting of the HA protein to lipid rafts, and to the apical membrane of polarized epithelial cells is thought to be mediated by regions within the transmembrane domain and in the exoplasmic region of the HA protein (Barman et al., 2001; Lin et al., 1998). In addition, the cytoplasmic tail domain of HA proteins may also have some function in localization of HA to lipid rafts (Zhang et al., 2000). Expression of HA alone can lead to HA protein release from the cell (Chen et al., 2007), possibly in the form of VLPs.

M1 trafficking to the membrane may occur by either binding to microfilaments in the cytoskeleton (Avalos et al., 1997), or by binding to viral HA and NA (neuraminidase) as they are trafficked through the exocytic network (Ali et al., 2000). M1 has an inherent capability to bind to plasma membranes, which is not due to the hydrophobic regions within the protein (Akhnoukh et al., 1996; Ali et al., 2000), but potentially due to electrostatic interactions (Ruigrok et al., 2000). M1 by itself does not localize to any particular section of the cell membrane. However, when M1 proteins are coexpressed with HA and NA proteins, M1 interacts with the cytoplasmic tails of these proteins (Chen et al., 2007) and so, becomes localized to lipid rafts where these proteins are located (Ali et al., 2000; Scheiffele et al., 1997; Zhang et al., 2000). M1 can localize to the lipid rafts only in the presence of HA (Chen et al, 2007). In addition, the coexpression of M1 with HA or NA proteins increases the amount of M1 recruited to the cell membrane (Gómez-Puertas et al., 2000).

The M1 proteins can assemble into enveloped VLP in baculovirus or vaccinia virus expression systems (Gómez-Puertas et al., 2000; Latham and Galarza, 2001). However, the M1

protein alone cannot cause VLP formation in a plasmid based production system (Chen et al., 2007), and the release of VLPs in the other systems could be due to helper effects from transmembrane or other proteins expressed from the other viral genes. This could be due to some sort of helper function given by other proteins in the baculovirus or vaccinia system. Like several enveloped viruses, baculovirus gp64 is also trafficked into lipid rafts on the cell surface (Haines et al., 2009), and this could have allowed M1 expression to cause budding of influenza VLPs.

Since viral membranes are enriched in cholesterol and glycosphingolipids, virions probably bud from these lipid rafts where all components of the virus are assembled, mostly on the apical surface of epithelial cells (Nayak et al., 2009; Scheiffele et al., 1999; Zhang et al., 2000). It is likely that the lipid rafts serve as a region where viral proteins are concentrated to levels sufficient to induce budding (Takeda et al., 2003). This requires the action of several viral and host factors and has been reviewed elsewhere (Nayak et al., 2009). Evidence points to virion morphology (spherical vs filamentous) being influenced by the polypeptide sequences of the membrane bound proteins, particularly the cytoplasmic tail domains of the HA and NA proteins (Jin et al., 1997), and the M1 protein (Chen et al., 2007), as well as by the action of the cytoskeletal network (Simpson-Holley et al., 2002).

Comprehensive reviews of the influenza virus production process from cells are found elsewhere (Nayak et al., 2009)

2.9.2. Influenza VLP production

Influenza VLPs have been produced in mammalian cells such as the COS-1 cell line (Gómez-Puertas et al., 1999; Gómez-Puertas et al., 2000; Mena et al., 1996) and HEK293 cells (Thompson et al., 2015; Venereo-Sanchez et al, 2016). However, BEVS has been increasingly

used to produce influenza VLPs. One of the factors that makes this system suitable for the production of influenza VLPs is the lack of sialyation in most insect cells (Altmann et al., 1999; Jarvis and Finn, 1995; Kulakosky et al., 1998) due to which HA glycoproteins on the surface of influenza VLPs will not bind to cells, and therefore, do not require the presence of viral NA to cleave VLPs off the cell surface, as in the case of mammalian cell lines (Gómez-Puertas et al., 1999). In the BEVS, multi-subunit influenza VLPs were first produced using a baculovirus coexpressing four influenza proteins, the hemagglutinin (HA), neuraminidase (NA), matrix (M1), and M2 proteins, with at least three of these (the HA, NA and M1 proteins) being incorporated into the virus-like particles produced (Latham and Galarza, 2001). Further studies have shown that VLPs produced using baculovirus vectors co-expressing HA, NA and M1 proteins have been able to produce protective immunity from influenza viruses (Bright et al., 2007; Pushko et al., 2005; Pushko et al., 2007), including against the H5N1 influenza virus (Mahmood et al., 2008). This has also been shown with particles containing only the HA and M1 proteins produced by co-infection of insect cells with baculovirus coding for the individual proteins (Quan et al., 2007). In addition, these particles were shown to be budded off the surface of the insect cells with size and morphology similar to wild type influenza virus produced from mammalian cell culture. Furthermore, the VLPs when administered result in protection against several strains of influenza virus (Quan et al., 2007). An extension to this work has been the incorporation of multiple HA serotypes into a single VLP, which led to the inhibition of replication of the two strains of virus under consideration (Quan et al., 2008). Work on influenza VLP production in the baculovirus insect cell system has led to the development of a series of pandemic and seasonal influenza vaccines from Novavax, which were in Phase 1 and Phase 2 trials respectively, when this thesis was written (Novavax website, March, 2016).

Chapter 3 : Temporal Characterization of Protein Production Levels from Baculovirus Vectors Coding for GFP and RFP Genes under Non-Conventional Promoter Control

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3.1 Abstract

The ease of use and versatility of the Baculovirus Expression Vector System (BEVS) has made it one of the most widely used systems for recombinant protein production However, co-expression systems currently in use mainly make use of the very strong very late *p10* and *polyhedron* (*polh*) promoters to drive expression of foreign genes, which does not provide much scope for tailoring expression ratios within the cell. This work demonstrates the use of different *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) promoters to control the timing and expression of two easily traceable fluorescent proteins, the enhanced green fluorescent protein (eGFP), and a red fluorescent protein (DsRed2) in a BEVS co-expression system. Our results show that gene expression levels can easily be controlled using this strategy, and also that modulating the expression level of one protein can influence the level of expression of the other protein within the system, thus confirming the concept of genes "competing" for limited cellular resources. Plots of "expression ratios" of the two model genes over time were obtained, and may be used in future work to tightly control timing and levels of foreign gene expression in an insect cell co-expression system.

Keywords: co-expression, baculovirus expression vector system, BEVS, insect cell, reporter protein, infection

3.2 Introduction

The baculovirus expression vector – insect cell system is not only versatile for expressing significant quantities of proteins requiring post-translational modifications quickly; it is gaining significant traction as a production platform for complex biologics. Both the ability of the insect cells to receive multiple baculoviruses carrying different transgenes and/or the ability of the baculovirus vectors to carry multiple transgenes have made the system a darling of those studying and producing virus-like particles or products requiring the expression of 'helper' proteins (Sokolenko et al., 2012). No matter the product requiring multiple proteins, it is unlikely that each protein needs to be expressed at the same level. Tailoring gene expression ratios is important in cases where the levels of proteins affect the composition of the final product (Tsao et al., 1996), or when the expression of certain proteins has a detrimental effect on the cells (Urabe et al., 2002). Furthermore, it can be argued that producing an excess of certain proteins is just a waste of cellular resources that could be otherwise channelled into useful product.

While there exists a number of studies describing polycistronic baculovirus vectors (Fitzgerald et al., 2006; Fitzgerald et al., 2007a; Kanai et al., 2013), controlled gene expression ratios have most predominantly been achieved by co-infection. While co-infection offers the flexibility of manipulating gene expression ratios by varying the multiplicity of infection (MOI) of individual baculovirus populations, the probabilistic nature of infection (Licari and Bailey, 1992) means that not all cells will receive the same proportion of the transgenes in each cell. Co-expression systems generally make use of the very strong very late *p10* and *polyhedron* (*polh*) promoters to drive expression of foreign genes. However, this does not provide much scope for tailoring gene product ratios within the cell. Given what is already known about baculovirus promoters and associated genetic elements from studies on: the *ie1* promoter (Jarvis et al., 1996);

the *basic* promoter (Bonning et al., 1994; Chazenbalk and Rapoport, 1995; Higgins et al., 2003); the *gp64* promoter (Grabherr et al., 1997); the Pcappolh, a hybrid of the *vp39* capsid and *polh* promoters (Thiem and Miller, 1990); tandem *ie1* promoters (Kojima et al., 2001); synthetic early promoters (Blissard et al., 1992), truncated *p10* and *ie1* promoters (Urabe et al., 2002; Urabe et al., 2006) and constitutive insect promoters like the *hsp70* promoter (Lu et al., 1996; Prikhod'ko et al., 1998); there is a dearth in the characterization of expression when these promoters are used in conjunction with the expression of a second gene under the control of a different promoter. At best, there are a few studies such as one by Urabe et al. (Urabe et al., 2002) for the production of AAV2 vectors, and the subsequent work on that system (Aucoin et al., 2006b; Aucoin et al., 2007; Kohlbrenner et al., 2005; Meghrous et al., 2005). In addition two recent patents have been filed on the use of weaker baculovirus promoters to drive the expression of non-structural components for the production of virus-like particles (Hu and Lin, 2013; Oker-Blom and Summers, 1992), and there has been some work on an early baculovirus detection system utilizing an early promoter (Dalal et al., 2005).

Beyond tailoring expression levels, there is also the ability to control the temporal nature of the expression with these promoters. Most characterizations of the promoters, when used in conjunction with another promoter-gene element, simply look at the resulting production levels and do not acknowledge that the 'sequence' of events may also play a role in the overall product formation. It is also possible that by taking advantage of sequential gene expression, there is an advantage over trying to express all of the different elements at once. While there is some evidence that suggests that there is no resource limitation when expressing multiple proteins at once (Berger et al., 2004), there is other evidence which suggests that competition may be a real effect. It has been shown that the deletion of the very-late *p10* gene causes improved production

from genes under the control of the very-late *polh* promoter (Chaabihi et al., 1993; Hitchman et al., 2010). Further evidence of competition comes from studies where co-expressing more than one chaperone protein actually caused a decrease in yield of the target protein of interest (Kato et al., 2005; Tate et al., 1999).

This work aims to demonstrate the use of different promoters in a polycistronic baculovirus to control the timing and expression of two easily traceable fluorescent proteins, the enhanced Green Fluorescent Protein (eGFP herein referred to simply as GFP) and a red fluorescent protein (DsRed2 herein referred to simply as RFP). The RFP gene is placed under the control of the very-late *polh* promoter, while the GFP gene was placed under the control of early (*ie1*), late (*basic*, *gp64* and *vcath*) and very-late (*p10*) promoters.

3.3 Materials and Methods

3.3.1 Cell culture and baculovirus production

Spodoptera frugiperda clonal isolate 9 (Sf9) cells (GIBCO, Carlsbad, CA, USA) were maintained in capped glass Erlenmeyer flasks in Sf900III media (GIBCO, Carlsbad, CA, USA) at a temperature of 27 °C on an orbital shaker rotating at 130 rpm. Maintenance cell cultures were kept at a density between 0.5 and 4×10^6 cells/ml.

3.3.2 Baculovirus construct generation

Five baculovirus constructs co-expressing GFP and RFP fluorescent proteins were generated for this work (Table 3-1). The GFP and RFP genes were isolated from plasmids pcDNA3-GFP and pCALNL-DsRed obtained from Addgene (Cambridge, MA, USA) using primers GFPF, GFPR, RFPF and RFPR (Table A-1) using a Phusion® High-Fidelity polymerase (Thermo Scientific,

Waltham, MA, USA). These genes were then cloned into baculovirus transfer vector pAcUW51 (BD Pharmingen, San Diego, CA, USA) using overlap extension PCR as described in other works (Bryksin and Matsumura, 2010). The resulting plasmid contained the GFP gene under control of a baculovirus *p10* promoter and the RFP gene under the control of a baculovirus *polyhedrin* promoter. The *p10* promoter was then replaced by four other AcMNPV promoters (*ie1, basic, gp64* and *vcath*), using overlap extension PCR to create the vectors for the five viruses used in this work. The viruses were generated from these vectors using the BD BaculoGold™ Transfection Kit (BD Pharmingen, San Diego, CA, USA). The primers used to isolate the promoters, and allow for the insertion of these promoters into p10-GFP-RFP are given in Table A-1. Two additional monocistronic viruses were created expressing either GFP or RFP. The genes were isolated as before using primers listed in Table A-1, and then inserted into the plasmid pFastBac1 (Life Technologies Inc, Burlington, ON, Canada) using the EcoRI and BamHI sites in the MCS. The primers used for these constructs are also listed in Table A-1. Viruses were generated from these constructs using the Bac-to-Bac® Baculovirus Expression System (Life Technologies Inc, Burlington, ON, Canada).

Table 3-1: Baculovirus constructs used in the experiments shown in this paper. All baculoviruses have been generated from pAcUW51. These constructs are referred to in the paper by the names given in ().

Construct Name	Description			
	Promoter 1	**Gene 1**	**Promoter 2**	**Gene 2**
p10-GFP-RFP (p10)	*p10*	GFP	Polh	RFP
ie1-GFP-RFP (ie1)	*ie1*	GFP	Polh	RFP
basic-GFP-RFP (basic)	*basic*	GFP	Polh	RFP
gp64-GFP-RFP (gp64)	*gp64*	GFP	Polh	RFP
vcath-GFP-RFP (vcath)	*vcath*	GFP	Polh	RFP
polh GFP	*polh*	GFP		
polh RFP	*polh*	RFP		

3.3.3 Baculovirus Amplification

Cultures were seeded at 0.5×10^6 cells/ml, allowed to grow to ~3×10^6 cells/ml to reach a mid-exponential phase, diluted to 1×10^6 cells/ml in fresh media and infected with various viruses. The cultures were allowed to proceed until the viability dropped to 70–80%, after which the culture medium was harvested and centrifuged at 1000 × g for 10 min in order to spin down cells and cell debris. The supernatant was removed and used as baculovirus stock for experiments.

3.3.4 Baculovirus Quantification

Virus samples were quantified by four different methods: end point dilution assay (Reed and Muench, 2938; King and Possee, 1992), real time PCR (George et al, 2012), flow cytometry (Shen et al, 2002), and a growth cessation assay (see Supplementary information for more detail) . The titres obtained from the end point dilution assay were used to determine the quantity of virus to add to the cultures.

3.3.5 Co-expression experimental design

35 ml cell cultures at a density of 1×10^6 cells/ml were infected with P2 stocks of the various viruses at MOIs of 5 or 25, in triplicate. Sampling was conducted at 0, 4, 8, 12, 16, 24, 36, 48, 72 and 96 hours, to capture both early and late events in the course of infection. Cell counts were conducted at each of these time points using a hemacytometer to determine cell density and viability. Samples were centrifuged at 300 × g for 7 minutes, after which the cell pellets were resuspended in a 2% solution of formaldehyde (Thermo Scientific, Rockford, IL, USA) in PBS and kept at 4ºC for one hour before analysis by flow cytometry. Controls included uninfected cultures, and cultures infected with monocistronic baculovirus vectors containing either GFP or RFP under the control of the polh promoter.

3.3.6 Flow cytometry analysis

Flow cytometry was conducted on a BD FACS Calibur (BD Biosciences, San Jose, CA, USA). The results were analyzed using FlowJo (Tree Star, Ashland, OR, USA). The threshold was based on FSC and was set at a value of 52, and served to remove small low scatter events. Green fluorescence was detected by the FL1 detector (emission 530, bandpass 30nm) and red fluorescence by the FL3 detector (emission 670 nm, longpass). All samples were analysed using a high flow rate (60 $\pm$ 3 µl/min). Compensation was conducted to remove signal overflow from GFP fluorescence into the FL3 channel and was set at 11.5% of the FL2 signal based on the level of fluorescence detected when cells were infected with a monocistronic vector containing the GFP gene.

3.4 Results

3.4.1 Virus stocks, progression of infection and effect on cells

Infection of the cultures with an MOI of 5 resulted in a synchronous infection as seen by growth cessation (Figure 3-1). The rate of decrease of viability in different cultures between 24 and 96 hours post infection (hpi) was different for the different constructs tested, with the constructs expressing the high levels of foreign protein overall showing higher rates of viability decrease than the ie1 and basic constructs which drove lower levels of foreign protein production and showed lower rates of viability decrease (Figure 3-1C)

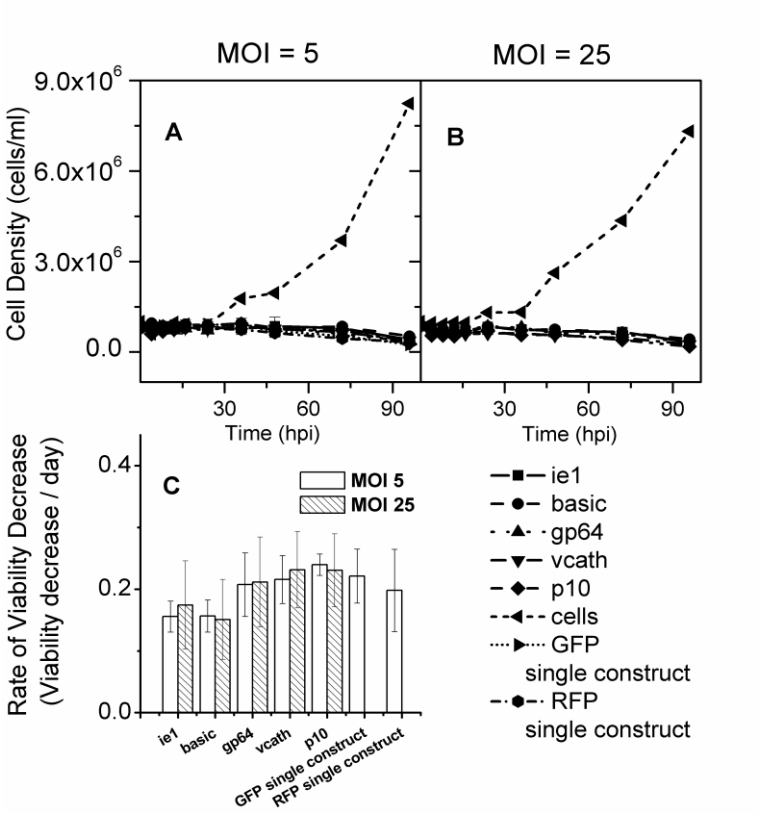


Figure 3-1: Cell density and viability of infected cell cultures: Figures 3-1A and 3-1B show cell density during the course of an infection experiment for cultures infected at MOIs of 5 (Figure 3-1A) and 25 (Figure 3-1B), obtained from hemacytometer counts. 35 ml cultures were infected with the following baculoviruses in triplicate: p10, ie1, basic, gp64 and vcath. Figure 3-1C shows the rate of viability decrease of the different constructs between 24 and 96 hours post infection. The points shown in the plots are the average of values for triplicate flasks and error bars represent 1 standard deviation above and below means.

To probe this further, and to ensure that the vectors were not compromised by using the alternative promoters to drive GFP, we followed the progression of the virus infection (MOI of 5 and 25) by monitoring the levels of baculovirus in the supernatant using real time PCR (Figure 3-2). No significant differences were observed in the replication of different constructs at MOI 5 or 25 (Figure 3-2A and 3-2B). In addition, no differences in replication-capable progeny were observed by the end-point dilution assay (Figure 3.2C). Taken together, these results are reassuring for two reasons: 1) the amount of virus taken up did indeed allow for synchronous infection in all cases based on the immediate cessation in cell growth; and, 2) ~ five times as much virus was taken up for a five-fold increase in MOI (Table 3-2).

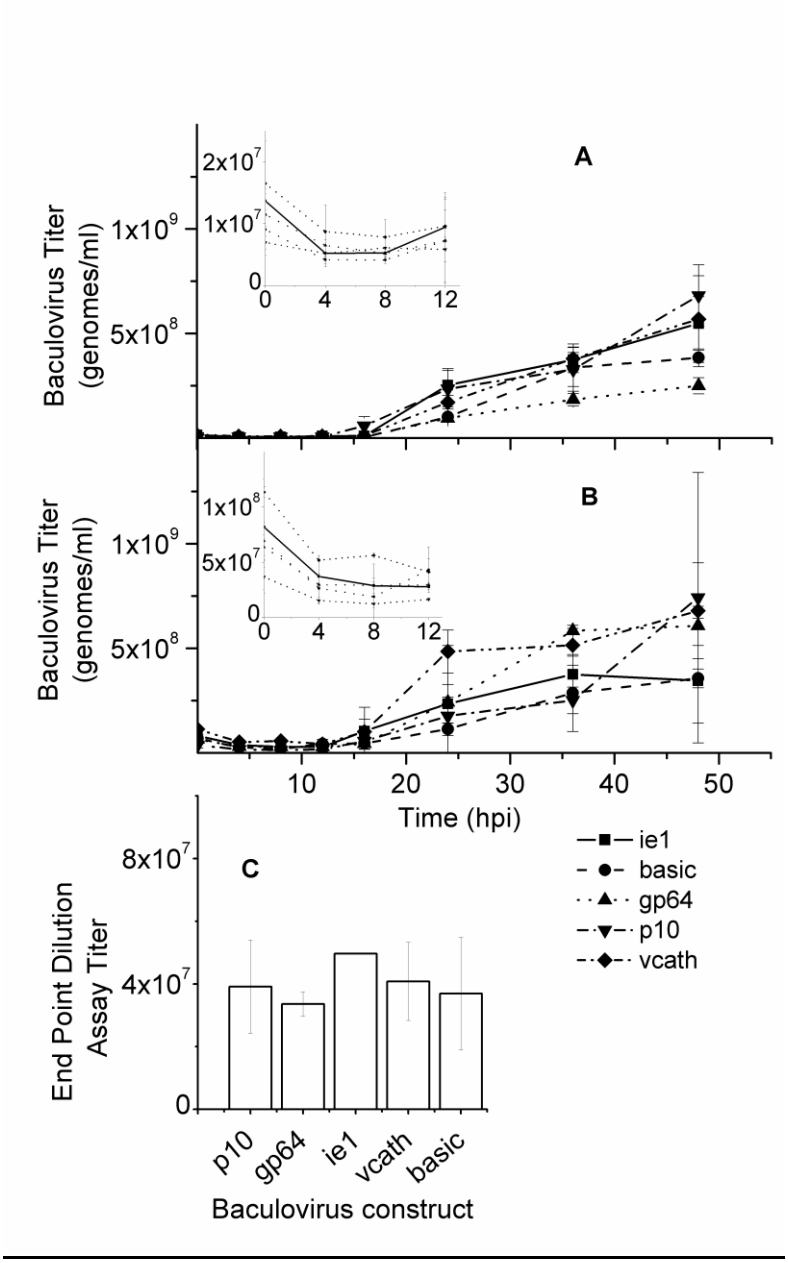


Figure 3-2: Baculovirus counts in cell culture supernatants over time, as determined using real-time PCR to determine genome counts (Figures 3- 2A and 3-2B) and the end point dilution assay to determine replicative virus titer (Figure 3-2C). The genome counts were performed for cultures infected at MOI 5 (Figure 3- 2A) and MOI 25 (Figure 3-2B). Inset figures show genome counts at early time points post infection. Figure 3-2C shows titers of replication competent baculovirus at 48 hours post infection for the various cultures infected at an MOI of 5.

Table 3-2: Average percent of vector taken up in co-expression experiments at MOIs of 5 and 25. Values were calculated by comparing vector genome titers in the supernatant at 0 and 4 hours post infection by real-time PCR (see supplementary information for details on methodology)

Vector	% Vector/Number of Vector Particles Taken Up By Cells			
	MOI = 5	MOI = 5	MOI = 25	MOI = 25
p10	25%*	1.76×10^{6}	58%	2.13×10^{7}
ie1	62%	8.43×10^{6}	55%	4.47×10^{7}
basic	54%	4.86×10^{6}	62%	4.28×10^{7}
gp64	43%	4.95×10^{6}	53%	3.39×10^{7}
vcath	47%	7.77×10^{6}	54%	6.15×10^{7}

***note: this value was obtained from triplicate cultures. A value this low has not been reproduced since.**

3.4.2 GFP/RFP Production

The main objective of this work was to observe the expression patterns resulting from infection with baculoviruses having foreign genes controlled by non-conventional promoters. While the cells seemed to be mostly in a single population for the first 36–48 hours of infection, the formation of a secondary population can be seen starting at 48, becoming more apparent at 72 and increasing further at 96 hours post-infection (two examples of which are seen in Figure 3-3). The number of events in the higher fluorescence population was found to correlate well with the overall viability of the culture (supplementary information). In addition, when the cultures were imaged by an ImageStream^{X} Mark II imaging flow cytometer (Amnis Corporation, Seattle, WA, USA), the lower fluorescence population was found to be mis-shapen cells that seemed to be dying or dead (data not shown). The fluorescence of the high fluorescence population was used for subsequent analysis.

Figure 3-4 shows the relative overall levels of green and red fluorescence observed for MOI 5 and 25 infected cells over time. The start of GFP production varied depending on the promoter in front of the gene, with the earliest signals at an MOI of 5 emerging after 4 hours under *ie1*; 8-12 hours under *basic*, and *gp64* control; 12-16 hours for *p10*; and 16-24 hours for vcath (Table 3-3). Increasing the MOI to 25 caused the GFP signals from the basic and gp64

constructs to emerge at 4 hours, while the time of emergence in the other constructs was not affected. Of the constructs, the highest levels of GFP, as well as the fastest increase in GFP expression level was seen when the gene was placed under the control of the *basic* promoter. The heavily delayed emergence of any fluorescence signal when using the vcath vector could be due to the very weak strength of the *vcath* promoter, and not necessarily an indication of the start of vcath expression. GFP expression in the GFP single construct emerged only at 16–24 hours post infection.

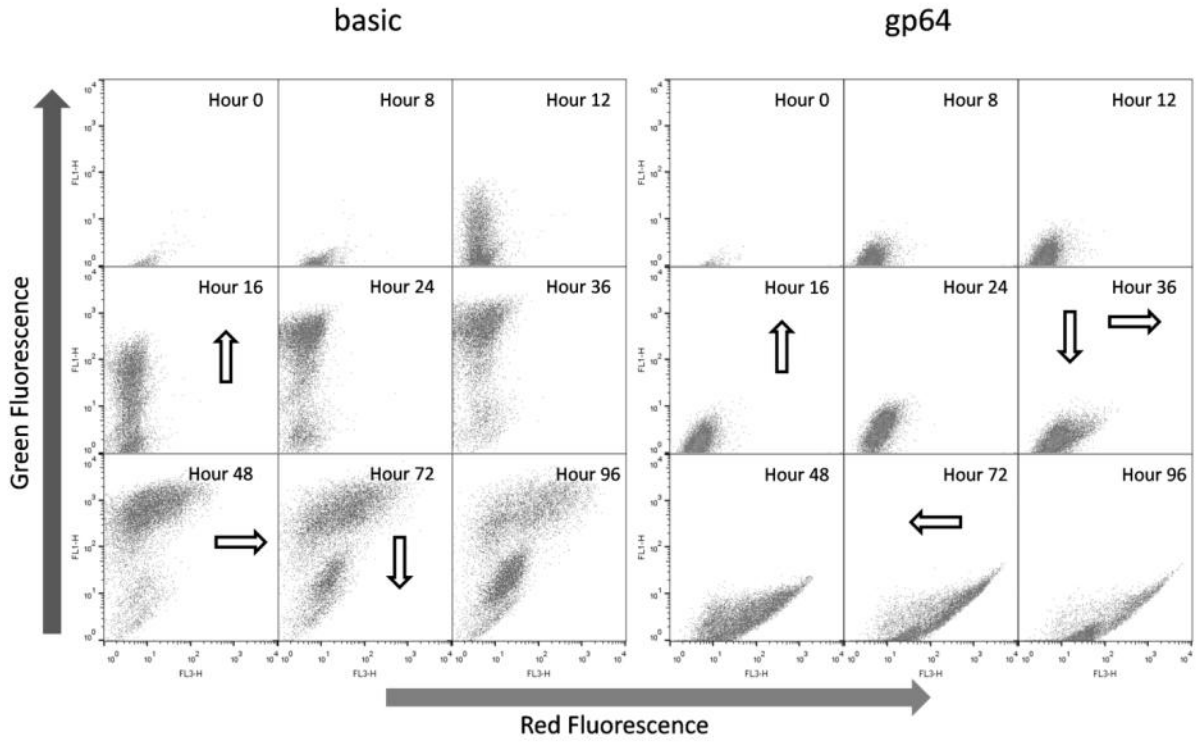


Figure 3-3: Representative flow cytometer scatterplots showing population distributions in cultures infected with basic and gp64 constructs over time. The plots compare values of red (FL3) and green (FL1) fluorescence over time post infection. Arrows indicate movement of populations as infection progresses.

Table 3-3: Times of detectable fluorescent protein detection.

Vector	Time at which protein production is detected (hpi)			
	GFP		RFP (polh)	
	MOI = 5	MOI = 25	MOI = 5	MOI = 25
Polh GFP*	NA	NA	16-24	NA
p10	12-16	12-16	16-24	24-36
ie1	4-8	4-8	16-24	16-24
basic	8-12	4-8	36-48	24-36
gp64	8-12	4-8	16-24	12-16
vcath	16-24	16-24	16-24	12-16
Polh RFP*	16-24	NA	NA	NA

***note: monocistronic baculovirus vectors with either GFP or RFP under the control of the polh promoter.**

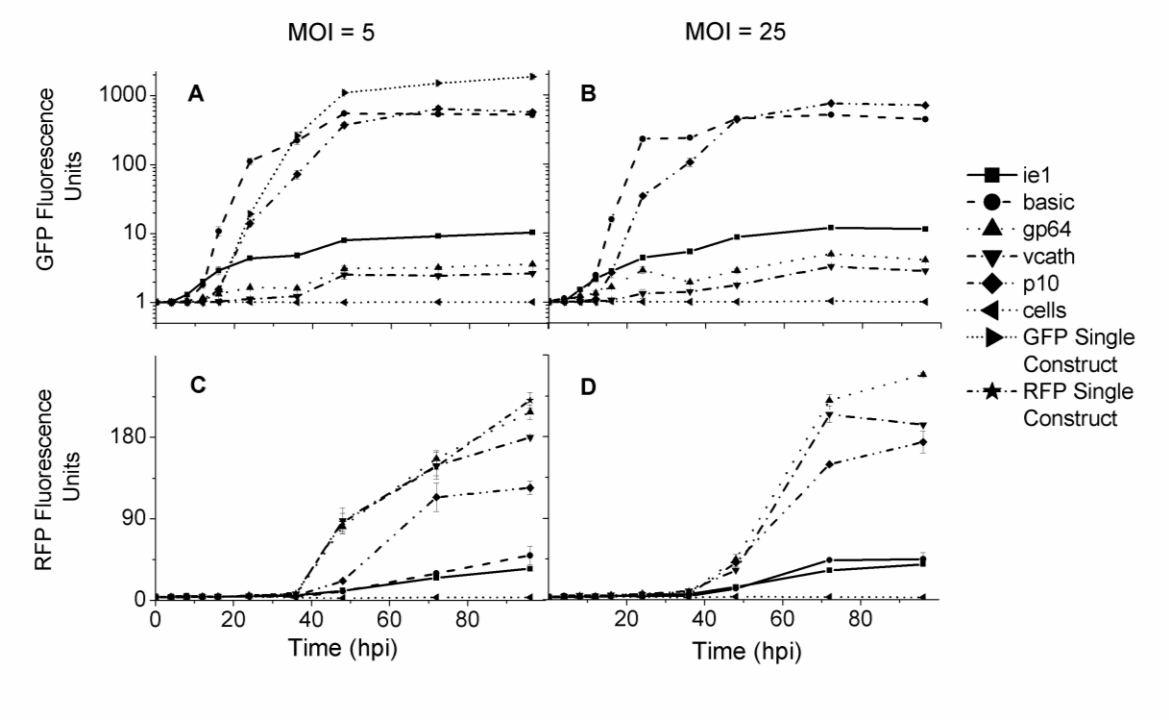


Figure 3-4: Expression of GFP and RFP From various baculovirus constructs: Relative expression levels of GFP in highly fluorescent living cells infected at MOI 5 and 25 (Figure 3-4A and 3-4B) and RFP (Figure 3-4C and 3-4D), with the p10, ie1, basic, gp64 and vcath, the polh-GFP and polh-RFP monocistronic vectors. The plotted values are obtained from triplicate flasks, and represent the geometric means of high fluorescence cell populations in cell cultures, and error bars represent 1 standard deviation above and below the mean

The choice of the promoter in front of the GFP gene also influenced the appearance of the signal from RFP, which was under the control of the polh promoter. At MOI 5, most constructs, including the RFP single construct showed an RFP signal starting at 16-24 hours post-infection.

However, the signal in the low GFP expressing vcath construct appeared much earlier (12–16 hours), while the high GFP expressing basic construct only showed an RFP signal at 36 hours post-infection, which could be due to the very high levels of GFP that were produced in this system early in the infection. Increasing the MOI to 25 had an almost universal effect of causing RFP signals to emerge earlier except for the p10 vector where RFP was detected later. The earlier emergence of signals with higher MOI was likely due to the increased number of viruses providing increased numbers of templates for protein production. Alternatively, the increased numbers of viruses may have caused increase early gene expression, thereby accelerating the progression of viral infection. The delay in RFP for the p10 construct may be the direct result of overexpression of the GFP gene.

GFP production showed minimal increases after 48 hpi in most cases, while RFP production generally increased till 96 hpi. It should be noted that at 36 hours, for many of the systems tested, there is a decrease in the GFP fluorescence – a dip of sorts – that may be indicative of a dual regulation of protein production or may be related to the onset of RFP production. Differences in RFP production from the different vectors were evident with the low GFP producing systems producing much higher levels of RFP (Figure 3-5), except for the ie1 construct. High levels of GFP production earlier in the infection cycle (basic construct) caused a significant reduction in the amount of RFP produced. Single constructs, with GFP or RFP under the control of the *polh* promoter, produced the highest levels of fluorescence in all cases.

The fluorescence levels of highly fluorescent cells infected with the single constructs were used to obtain a correlation of GFP to RFP fluorescence units, which ultimately allowed for the creation of protein production ratio profiles from the different polycistronic constructs (Figure 3-6). The figure shows that GFP and RFP production ratios vary widely between

constructs. In general, GFP was always produced at a lower level than RFP, except in cells infected with the *basic* promoter, where GFP was present at a level greater than that of RFP till 36 hours post infection, following which GFP was present at roughly half the level of RFP. The vcath and gp64 constructs produced far more RFP than GFP.

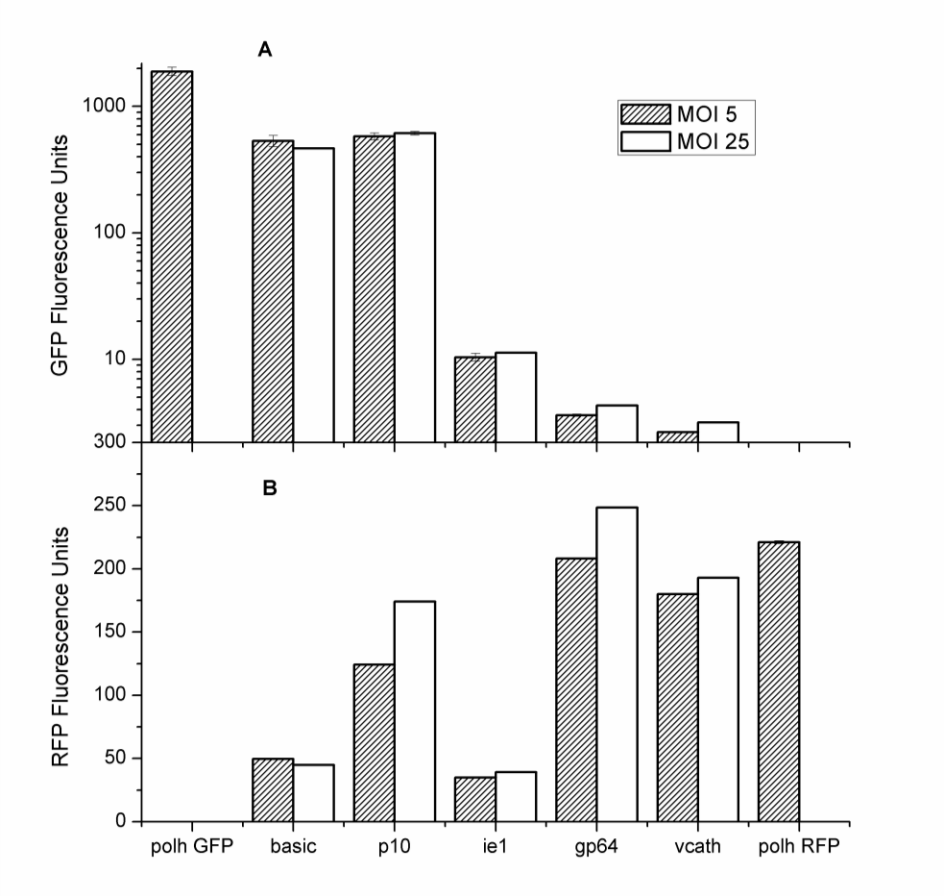


Figure 3-5: Peak levels of GFP and RFP produced from different constructs: Maximum levels of green (Figure 3-5A) and red fluorescence (Figure 3-5B) in cell cultures infected at MOIs 5 and 25 with the p10, ie1, basic, gp64 and vcath vectors, and the monocistronic polh-GFP and polh-RFP constructs. The plotted values are the averages of fluorescence means of cell populations, multiplied by the total number of events to give the "culture fluorescence" from triplicate flasks, and error bars represent 1 standard deviation above and below means.

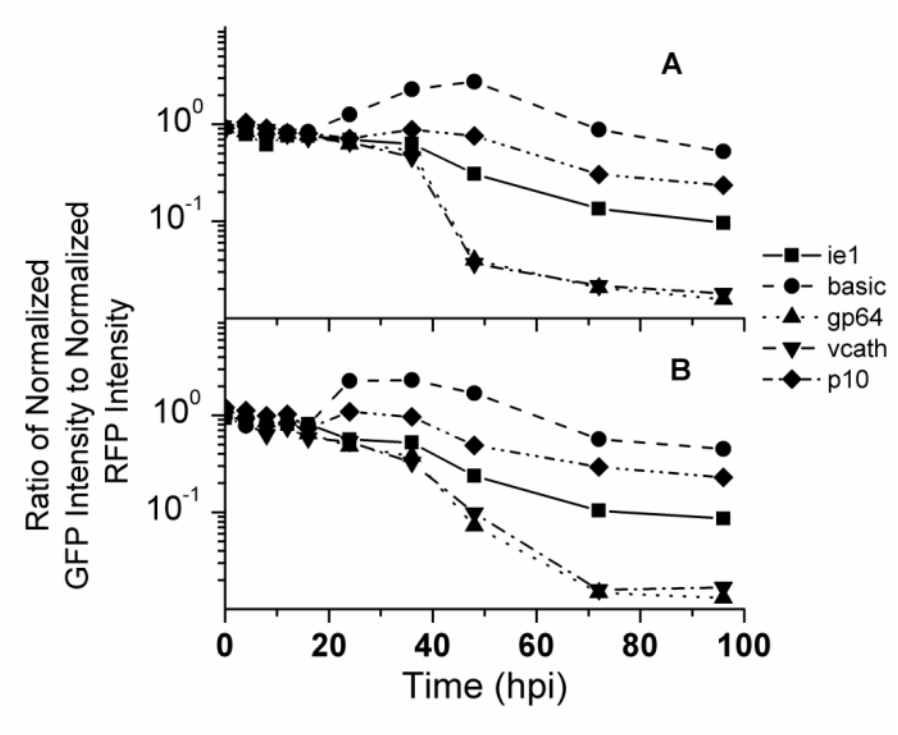


Figure 3-6: Expression ratios of GFP and RFP from different constructs: GFP/RFP expression ratio profiles over the course of cell culture infection with the p10, ie1, basic, gp64 and vcath vectors at MOIs of 5 (Figure 3-6A) and 25 (Figure 3-6B). Profiles were obtained by first converting the geometric means of GFP fluorescence values of highly fluorescent cells into RFP fluorescence values using the correlation obtained by comparing fluorescence values of the two monocistronic polh-GFP and polh-RFP constructs. The ratios of converted GFP and RFP values were then plotted.

3.5 Discussion

3.5.1 Vector Alteration: Changing Promoter Sequences and Effect on Vectors

For over twenty years researchers have alluded to the benefits of offsetting expression levels through promoter control (Tate et al., 1999), yet nobody has gone ahead and done a study to show how this should be done. Our belief is also that promoters can help stage an appropriate sequence of events that can lead to the efficient production of complex multi-protein molecules such as VLPs. This is in part based on evidence from natural systems such as influenza virus replication, where the rates of synthesis of different proteins differ over the course of the infection, with the Matrix (M) protein being produced at the highest levels after the other genes

have reached their peak production (Meier-Ewert and Compans, 1974). This could be due to the fact that the M protein which forms the inner layer of the influenza particle shell is only needed at later times during the virus replication cycle. The baculovirus system has an even more complex temporally separated protein production scheme with various transcription initiators being produced first, followed by structural proteins, with the outer protective proteinacious coats being produced only at the end (Rohrmann, 2011).

This work aims to demonstrate the utility of promoter control in a very simple two protein co-expression system where the proteins are not secreted and not believed to interact. The enhanced Green Fluorescent Protein (GFP) and DsRed2 Red Fluorescent Protein (RFP) were further chosen because of their green and red fluorescence emissions, which enabled easy and accurate tracking of their production levels in infected cell cultures. Five different coexpressing virus constructs were generated for this experiment. In each of these, the RFP gene was placed under the control of a *polh* promoter, which activates at high levels during the very late phase of infection, at about 27 hours post infection (Bonning et al., 1994). The promoter controlling expression of the GFP gene is varied between the five constructs: the *p10* promoter, which activates during the very late phase of infection, at about 19 hours post-infection (Bonning et al., 1994) the *basic* promoter, which is active from about 13 hours post infection (Bonning et al., 1994; Hill-Perkins and Possee, 1990); the *gp64* promoter, which activates in both the early phase (from 6 hpi) at low levels and during the late phase at much higher levels (from before 24 hpi) (Garrity et al., 1997); and the *vcath* promoter, which activates during the late phase at around 22 hpi (Hodgson et al., 2007; Hom et al., 2002; Slack et al., 1995a). Our results agree with these findings for the most part, however, GFP was observed earlier when controlled with the p10 and basic promoters, likely due to the sensitivity of the detection used in this study.

It is known that MOI can play a role in overall gene expression levels as well as onset of protein expression and so, extreme care was taken to have 'equivalent' MOIs for experiments comparing different baculovirus constructs at MOIs of 5 and 25. Although titers via the different methods differed as expected (Figure A-1), together the methods were a good indication of: the composition of the stocks; and the minimal interference to baculovirus replication that may have been caused by using promoters that also drive essential genes. Given the fluctuations in seeding (Figure 3-2) it is expected that an MOI of 5 actually ranged from 4.3 to 8.8 and an MOI of 25 actually ranged from 27 to 50.

The effects of 're-using' promoters that control the expression of native genes are not extensively documented. Virus replication is an important aspect of BEVS that could be affected by changing native expression patterns with the 're-use' of native promoters. To examine this, baculovirus concentration in the media were measured by real-time PCR over the course of infections (Figure 3-2A and 3-2B). A further experiment was also conducted analysing replicative baculovirus titres at 48 hours post infection (Figure 3.2C). Both methods revealed that baculovirus replication did not vary between constructs. It still remains to be seen if any of the constructs are more prone to the production of defective interfering particles upon repeated passaging.

3.5.2 Effect of Promoters and MOI on the Detection of Green and Red Fluorescent Protein

It was hoped and expected that GFP levels would vary given that its expression is being driven by different promoters. However, it was also found that the emergence of RFP also varied between constructs. For the most part, the times of RFP emergence was around 16 – 24 hours post infection, including for the single construct. However, the basic construct, which showed high levels of early GFP expression, showed an RFP signal very late post infection (36 hours).

The late emergence of the RFP signal is thought to be directly related to the amount of resources committed by the cell to producing GFP. The increase in MOI from 5 to 25 caused earlier emergence of RFP production, with production in the cultures infected with the basic, vcath and gp64 constructs showing signals up to 12 hours earlier. Of interest is the RFP fluorescence emanating from the vcath and gp64 virus infecting cultures, which can be seen at low levels as early as 12 hours post-infection. This further reinforces studies by others, including Hu et al. (Hu and Bentley, 2000), which shows that increasing MOI can significantly accelerate the progression of infection with an earlier activation of genes from the very late phase of infection.

3.5.3 Effect of Promoter on GFP and RFP levels: Competing for Resources

Although there was no evidence of interference in terms of baculovirus replication, the idea that we might be creating or alleviating 'competitive' environments with the use of these different promoters remained. The vcath and gp64 viruses produced the least amount of GFP, and the highest levels of RFP, while the basic construct, which expressed GFP at very high levels early during infection, produced far lower amounts of RFP than the p10 construct. These conclusions are further supported by data from the single constructs producing only GFP or RFP, which show greater levels of gene expression than any of the dual constructs (Figure 3-6A and C). In addition, co-infecting the two single constructs (overall MOI of 10) decreased the production of fluorescence molecules compared to cells infected with only the single foreign gene vectors, or cells infected with the p10 construct. The ie1 construct was an exception to the above observations, as it drove low levels of both GFP and RFP. It is thought that this may be related to some type of interference with the production of early transcriptional factors. Otherwise, this could be because of some form of regulation of the *polh* promoter by the spatially close ie1 promoter within the construct.

3.5.4 Effect of MOI on GFP and RFP levels: Competing for Resources

Many examples of 'production saturation' exist in literature. Excessive multiplicity of infections (MOI) have indeed lowered of the amount of recombinant protein produced in some systems as far back as a study done on the production of β-galactosidase where infection of a culture at an MOI of 50 resulted in a poorer performance compared with infections at MOIs of 0.05, 0.5 and 5 (Bedard et al., 1994). In our system, it was thought that the increase in MOI from 5 to 25 would increase the competition phenomenon, especially for those constructs with promoters driving late gene expression. For most of the constructs, there was very little improvement in the levels of GFP and RFP observed when increasing the MOI; however, despite the minimal increase in protein production, the results showed that there is a form of competition that arises from the expression of a secondary foreign protein. This was seen from the differing levels of RFP produced, despite always being under the control of the *polh* promoter. In the cases where there was infection with the vcath and gp64 constructs, significant increases in RFP levels with infection at the higher MOIs were observed, giving rise to the notion that the cell has a total protein capacity that can be utilized in many different ways depending on the promoter combinations used to drive gene expression and protein production.

3.5.5 Further Implications

This research also has implications for new high capacity multiprotein production systems such as the MultiBac system (Fitzgerald et al., 2006; Fitzgerald et al., 2007a), where nutrient limitations may be important when producing proteins at high levels. In these systems, multiple genes are expressed at very high levels under the control of very strong promoters, thereby competing for limited resources. The utility of these systems lies in their ability to express complex protein structures such as virus-like particles with several virus components,

and enzyme complexes to perform complex reactions. However, over-expression of some components could lead to sub-optimal protein ratios in the final product, and cause reduced expression of correctly assembled product containing appropriate ratios of component proteins. In addition, high expression of genes that are not needed in large quantities can lead to a "waste" of cellular resources that could be used for the production of other proteins. It is possible that in some cases, inappropriate protein ratios and wasted cell resources make it impossible to use this system for the production of complex proteins. This paper demonstrates the use of different promoters to control the timing and expression of genes within BEVS, which we believe is the next step in improving the system for the production of complex proteins. In addition to the large number of promoters available within the baculovirus genome, foreign protein production can be further modulated by the use of baculoviral and other regulatory elements such as homologous regions (HRs) (Guarino et al., 1986; Ishiyama and Ikeda, 2010), as well as non-baculoviral, and truncated (Urabe et al., 2002; Urabe et al., 2006), hybrid (Thiem and Miller, 1990) or tandem (Kojima et al., 2001) promoters that extend the duration of protein production, or change expression levels of native promoters. Artificial promoters (Blissard et al., 1992; Rankin et al., 1988) may also be used for modulating expression levels.

3.6 Conclusions

A detailed profile of expression levels over the entirety of the infection period by different vectors has been created and lends itself to serving as a template for "designing" expression systems in which the timing and expression level of different proteins can help in the optimization of product formation. Furthermore, this work shows that the expression of a gene under the control of one promoter can influence the production of protein whose gene was

controlled by another. Only one construct did not adhere to this theory: the ie1 construct. The latter resulted in low levels of both GFP and RFP, a result attributed in part by the fact that the *ie1* promoter is recognized by a different polymerase, namely DNA polymerase II. In addition, this work should serve to stimulate further research into the use of other baculovirus promoters, enhancer regions, and non-native promoters and other control elements within the baculovirus insect cell system, which can be characterized to enhance the ability to rationally design the 'expression' system.

Chapter 4 : Tracking RNA Expression Levels from Baculovirus Vectors Encoding for GFP and RFP

4.1 Introduction

The work presented in Chapter 4 (George et al., 2015) showed that promoters can be used to achieve fine and predictable control of gene expression within the baculovirus insect cell system, and that genes within this system do, in fact compete with each other for resources. However, the process of protein production involves both transcription and translation, and the previous work only examined the protein levels of two fluorescent proteins (enhanced Green Fluorescent Protein, referred to as GFP, and red fluorescent DsRed2, referred to as RFP) co-expressed within cells in the baculovirus insect cell system. Therefore, there is no information on how closely translation is related to transcription, and if the effect of competition is present at the transcription or translation levels, or if each of these individual processes is a contributing factor. Therefore, in this study, we tracked the levels of intracellular GFP and RFP RNA over time, from samples that were isolated at the same time, and from the same cultures as those used for the previous work. This enabled us to directly compare RNA and protein expression levels over time. This was done by semi quantitative real time PCR (qPCR), in which levels of GFP and RFP RNA were compared to levels of a control to get a value in terms of "fold difference" over levels of control RNA. As described in the previous chapter, the different viruses used contain the RFP gene under the control of the very-late *polh* promoter while GFP control is varied between the early (*ie1*), late (*basic*, *gp64* and *vcath*) and very-late (*p10*) promoters. These viruses are referred to as the ie1, basic, gp64, vcath and p10 viruses, and samples of cells infected with these viruses are called ie1, basic, gp64, vcath and p10 samples.

One of the aims of the work was to track levels of GFP and RFP transcripts using the insect cell 28S rRNA levels as a control, to control for variability in amounts of RNA extracted from cell pellets. The use of 28S rRNA as a control has been investigated in the BEVS system, and has been shown to be superior to several other commonly used controls in terms of transcript level maintenance over an infection cycle (Xue et al., 2010). In addition, the relative abundance of this RNA makes is quite easy to detect, as opposed to other low abundance control transcripts such as TATA box binding protein (TBP) gene (Xue et al., 2010) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Soos et al., 2011). Despite these reports, no correlation of 28S rRNA transcript levels with respect to cell density has been shown and experiments do not demonstrate the robustness of this approach at varying multiplicities of infection and culture densities. Therefore validation of 28S rRNA as a control was tested over a wide range of cell densities and during infection at several MOIs.

4.2 Materials and Methods

RNA transcript levels of the different genes of interest (*DsRed2* and *eGFP*) and control gene levels (28S rRNA) were tracked during the baculovirus infection process to monitor transcription patterns under the control of various promoters. The samples used were obtained from the same cultures as those used in the previous work on tracking fluorescent protein levels in cells over time (Chapter 3).

4.2.1 RNA extraction

RNA extraction from cell samples was conducted by spinning down cell culture samples at 300 × g for 7 minutes to separate the cells from the media. Cell pellets were then stored at -80°C for

further treatment. Cell pellets were then subjected to RNA isolation using TRIzol® reagent (Life Technologies Inc., Burlington, ON, Canada), according to the protocols recommended by the manufacturer. All samples were treated with 5 μg RNase-free glycogen to enhance RNA yield, especially from low cell density samples. Extracted RNA samples were stored at -80°C for reverse transcription.

4.2.2 Reverse transcription

Reverse transcription to synthesize cDNA from extracted RNA samples was conducted using the reverse primers 28SrRNA R, RT GFP-R and RT RFP-R in Table 4-1. The levels of control 28S rRNA was tracked by using a previously described primer (28SrRNA R) for reverse transcription (Xue et al., 2010, Table 4-1). Reverse transcription of the GFP and RFP transcripts were conducted using primers RT GFP-R and RT RFP-R specific to regions in these (Table 4-1). All primers used in this work are listed in Table 4-1. Reverse transcription was performed using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Burlington, ON, Canada) using a slightly modified manufacturers' protocols. Briefly, to avoid exceeding the cDNA conversion capacity of the kit, given the high abundance of template RNA, 2 μl of diluted extracted RNA was mixed with 2 μl of 10 × RT Buffer, 0.8 μl of 25 × dNTP mix, 0.8 μl of each of the reverse primers at a concentration of 25 μM, and 1.0 μl Multiscribe™ reverse transcriptase and then topped off with nuclease-free water, for a total reaction volume of 20 μl. The 28S rRNA samples were run separately with a 1/50 diluted RNA sample as template, as this was found to yield a very reliable result, while the GFP and RFP samples were run together in one tube with a 1/10 dilution of sample, which is necessary due to the much lower abundance of GFP and RFP transcripts in relation to 28S rRNA. The samples were then placed in a VeritiTM 96 Well Thermal Cycler (Applied Biosystems, Burlington, ON, Canada) and run at 25°C for 10 minutes,

37°C for 120 minutes and 85°C for 5 minutes. These reactions were then stored at -20°C until analysis by qPCR.

4.2.3 qPCR

qPCR reactions were conducted on a StepOne Plus Real-Time PCR system (Applied Biosystems, Burlington, ON, Canada). The determination of transcript levels for each of the transcripts was conducted in separate reactions. 28S rRNA levels were determined using previously described forward primer 28S-F and reverse primer 28S-R (Xue et al., 2010), which amplify out a region in the Sf9 28S rRNA. GFP and RFP transcript levels were determined using primers RT GFP-F and RT GFP-R, and RT RFP-F and RT RFP-R (Table 4-1). Each reaction consisted of 2 µl cDNA sample mixed with 10 µl of 2 × Power SYBR® Green PCR Master Mix (Applied Biosystems, Burlington, ON, Canada), forward and reverse primers at a final concentration of 900 µM, and nuclease-free water to a final volume of 20 µl. All reaction components except the cDNA were prepared as a master mix and mixed together with cDNA in a MicroAmp Fast Optical 96-Well Reaction Plate (Applied Biosystems, Burlington, ON, Canada). The plate was then sealed with MicroAmp Optical Adhesive Film (Applied Biosystems, Burlington, ON, Canada) and run according to reaction conditions described in the literature (Xue et al., 2010). The initial denaturation was run at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 5 seconds, annealing at 55°C for 20 seconds and extension at 72°C for 20 seconds. Melt curve analysis, for distinguishing the different species of DNA amplified during the reaction, was conducted by holding samples at 55°C for 10 seconds and then increasing the temperature in 0.3°C increments of 15 seconds to 95°C, with fluorescence being measured during the ramping stage.

The amplification curves obtained from qPCR were then analyzed by assumption-free analysis (Ramakers et al., 2003) using the program LineReg PCR (Ruijter et al., 2009). This method allowed the PCR efficiency of each reaction and the average efficiency of all similar reactions on the plate to be determined. The average efficiency was used to compare cycle threshold (Ct) values of different reactions on the same plate, thereby allowing for determination of fold difference in cDNA abundance between samples. RFP and GFP transcript levels were normalized to the 28S rRNA levels, which was made possible by implementing a common fluorescence threshold for all reactions on the plate. This allowed us to account for differences in TRIzol® RNA extraction between samples as well. Briefly, the formula for the quantity of unknown transcript was:

$$X_{0U} = X_{O\,28S}\frac{e^{(C_{T28S})}}{e^{e^{(C_{TU})}}} \quad \text{… Equation (1)}$$

And:

$$X \propto N \propto F$$

Where:

X_{OU} is the quantity (by mass) of either the GFP or RNA transcript cDNA present in the original cDNA sample; $X_{O\ 28S}$ is the quantity (by mass) of the 28S rRNA cDNA present in the cDNA sample; N is the number of moles of cDNA of any individual species present at the beginning of the reaction; F is the "initial fluorescence level" due to cDNA of any individual species at the beginning of the reaction; C_T is the cycle threshold number for each individual reaction.

Once the X_{OU} values have been obtained for GFP and RFP (by setting $X_{O\ 28S}$ to the arbitrary value of 1), the number of copies of the two species of cDNA in the original sample (N)

can be compared by dividing each of the X_{OU} values by the molecular weight of the amplified DNA fragment (38751.9 g/mol for GFP and 39218.3 g/mol for RFP).

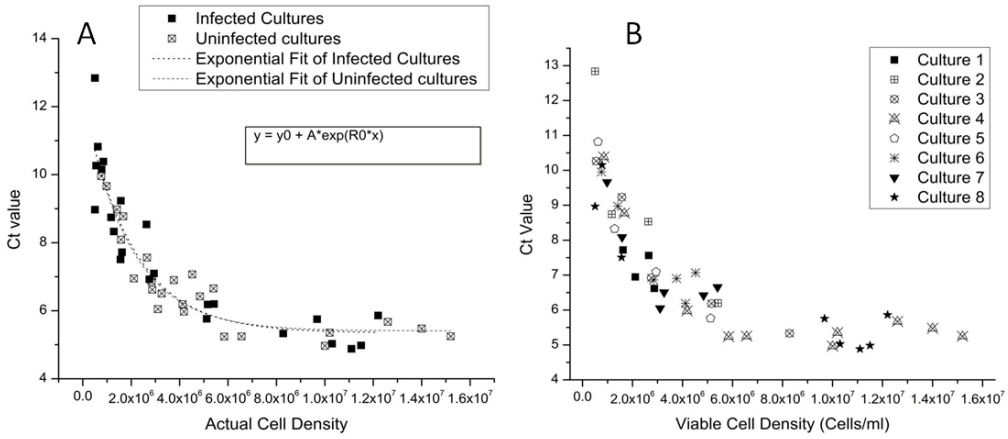
Table 4-1: List of primers used for tracking intracellular RNA levels

Primer Name	Sequence	Description
28SrRNA F	5′-CGA CGT TGC TTT TTG ATC CT-3′	Forward and reverse primers used in tracking 28S rRNA levels
28SrRNA R	5’ - GCA ACG ACA AGC CAT CAG TA – 3’	
RT GFP-F	5’ – CCT GAA GTT CAT CTG CAC CA – 3’	Forward and reverse primers used in tracking eGFP RNA levels
RT GFP-R	5’ – GAA GAA GTC GTG CTG CTT CA – 3’	
RT RFP-F	5’ – AAG CTG AAG GTG ACC AAG GG – 3’	Forward and reverse primers used in tracking DsRed2 RNA levels
RT RFP-R	5’ –CCC TTG GTC ACC TTC AGC TT – 3’	

4.3 Results

4.3.1 Validation of the use of 28S rRNA as a control gene

Eight random infected and uninfected cultures were sampled at different times, corresponding to a wide range of cell densities (~0.5 × 10^6 cells/ml to 1.5 × 10^7 cells/ml) with samples being infected by different viruses containing 2 different transgenes, grown at different volumes (ranging from 30 ml to 750 ml), and infected at MOIs ranging from ~0.2 to 5.



4-1: qPCR conducted on infected and uninfected cell cultures to detect 28S rRNA transcript levels (Figure4-1 A). Figure 4-1 B shows the same points grouped by culture number

Figure 4-1 shows the correlation between cycle threshold (Ct) values from the amplification of 28s rRNA cDNA, extracted and reverse transcribed from infected and uninfected cultures, and the cell densities of the cultures. As can be seen from the figure, the Ct values seem to vary exponentially with respect to the cell density, which is expected as Ct is related to the concentration of template in the reaction by the formula:

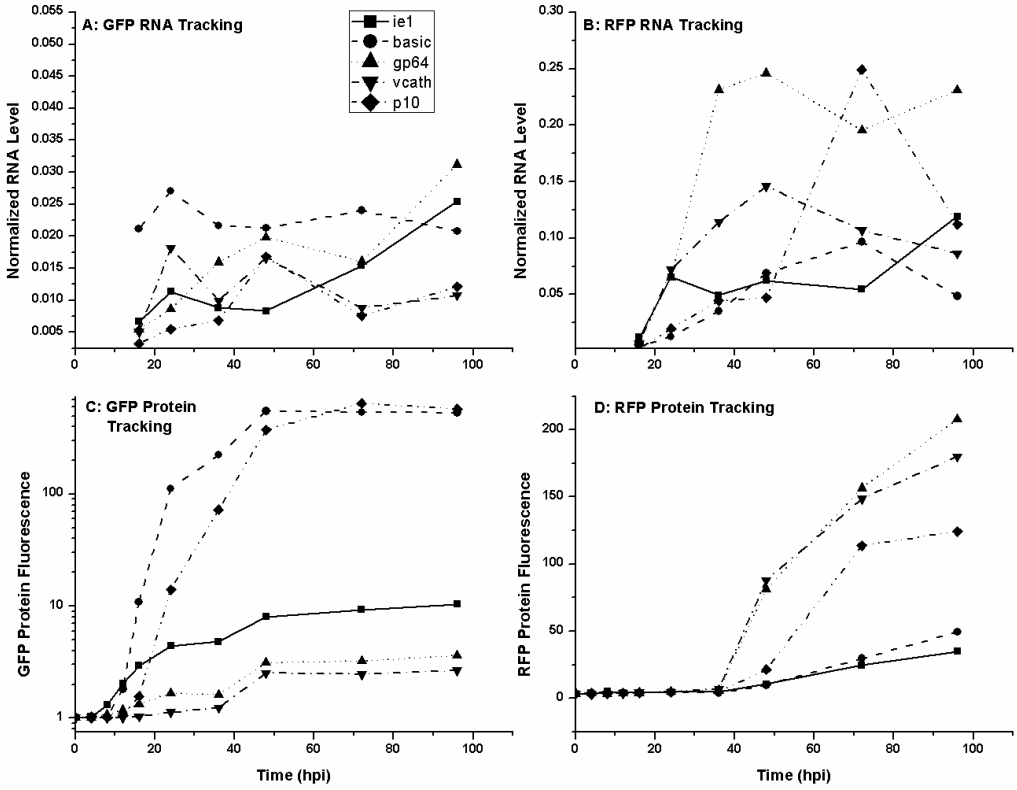
$$A_t = A_o * (1 + E)^{Ct} \quad \text{…Equation (2)}$$

where A_t is the copy number of the amplicon at the cycle threshold, A_o is the initial copy number, E is the efficiency of the reaction and Ct is the cycle threshold number. It is important to note that the fit of the points from infected cultures overlaps the points from the uninfected cultures, thereby indicating that 28S rRNA levels are density dependent and not a function of the infection of cells.

Figure 4-1B shows the correlation between cycle threshold (Ct) values and cell densities of different cultures at different densities. As can be seen, all cultures follow the same general trend.

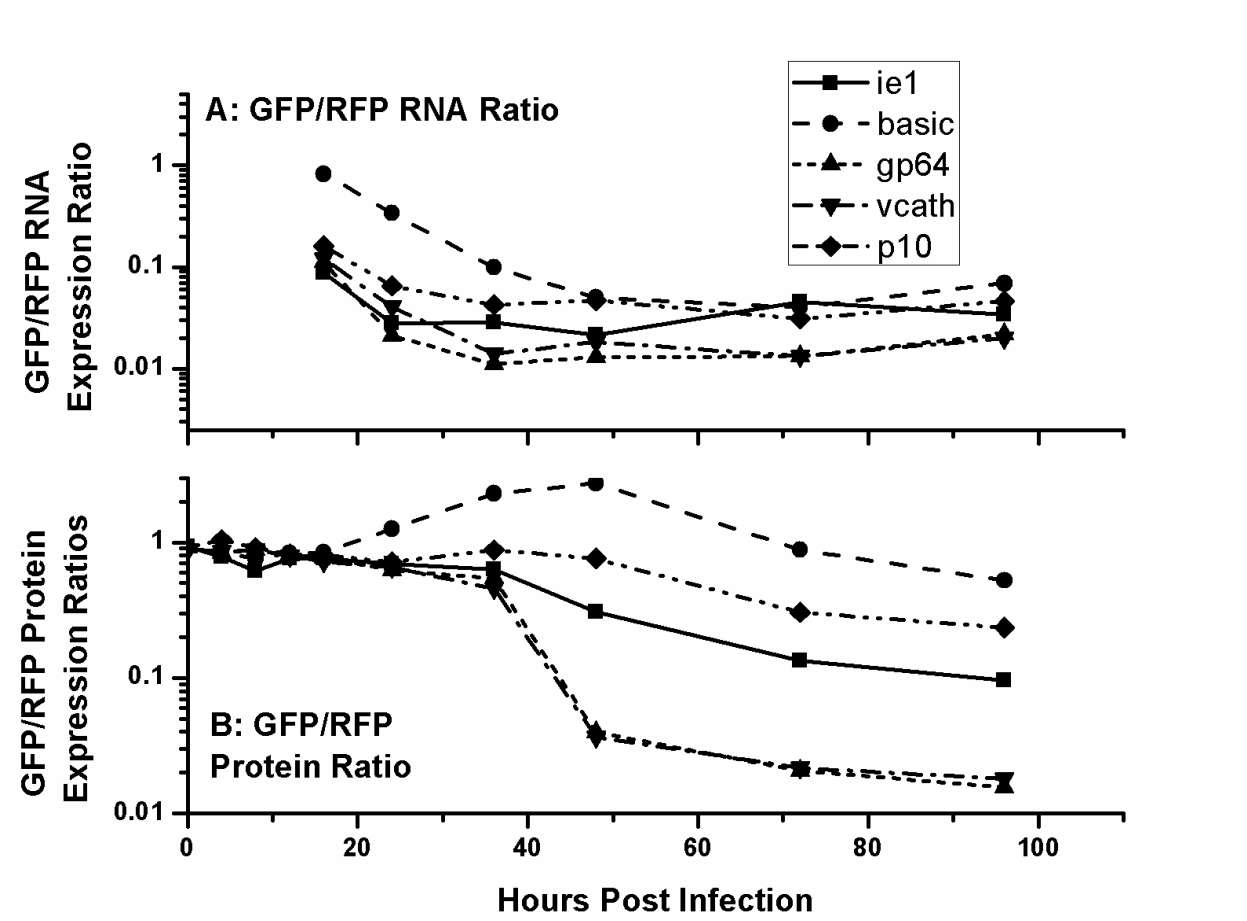
4.3.2 GFP and RFP tracking

Sf9 cells infected with GFP and RFP expressing baculovirus constructs were tracked using qPCR from time points 16 hours post infection (hpi). As can be seen in Figure 4-2, the levels of GFP and RFP are markedly different between constructs. Before 24 hours, the basic baculovirus infected cell samples seem to have much higher levels of GFP than other constructs. Past this time point, higher levels of GFP transcripts are seen to be produced from the p10 promoter. RFP RNA levels increase after 16 hpi in all constructs, but to different levels. In addition, the peak RFP RNA levels seems to be between 36 – 48 hours for most constructs except in the basic and p10 samples, where peak levels are only reached at 72 hpi. Interestingly, the RFP transcript levels when driven by the *ie1* promoter seem to be increasing even after 72 hours post infection.



4-2: GFP (4-2 A) and RFP (4-2 B) RNA levels in cells infected with different baculovirus constructs, normalized with respect to cellular 28S rRNA levels. Figures 4-2 C and 4-2 D show corresponding GFP and RFP protein expression levels.

Figure 4-3 shows the ratio of normalized GFP and RFP transcripts over time in each of the cell cultures infected with the different viruses. The level of GFP is generally lower than that of RFP at all time points except in basic samples at 16 hpi, where the ratio is close to 1. It is likely that ratios greater than 1 will be seen if basic samples are tracked before 16 hpi. The other constructs have GFP/RFP ratios of near 0.1 only at 16 hpi (the first recorded time point), after which they reduce drastically.



4-3: GFP/RFP RNA (4-5 A) and protein (4-5 B) ratios in cells infected with different baculovirus constructs

4.4 Discussion

The first step of this work was to validate the use of 28S rRNA as a control for RNA tracking, under a variety of different conditions. It can be seen that a correlation between the level of 28S rRNA and cell density exists. At higher cell densities, however, there was a greater amount of variability. This could be due to several factors, with the most likely being that the reverse transcription reaction was overloaded with template RNA at these densities, due to which the conversion efficiencies vary widely. Future work may consider lowering the amount of RNA used when doing the reverse transcription reactions for high cell density samples.

As can be seen from Figure 4-2, the levels of RNA in samples show patterns similar to the levels of fluorescent protein detected in previous work presented in Chapter 3 (George et al., 2015), which have been reproduced in Figure 4-2. For example, the early emergence of a GFP signal under the control of the basic promoter can be seen in both RNA (Figure 4-2 A) and protein production. RFP RNA levels (Figure 4-2 B) can also be generally related to fluorescence levels. The gp64 samples show the highest levels of RFP RNA and protein, while the basic samples show the least. One anomaly is the level of RFP RNA in the vcath construct infected cells, which were only higher than the p10 construct RFP RNA levels at one time point, while fluorescence data (George et al., 2015, Figure 4-2 C and D) shows that the RFP protein level from vcath samples was higher than in the p10 construct infected cells at almost all time points. This could indicate that the level of RNA in cells at earlier time points is especially influential in determining the level of protein being produced at subsequent time points. Alternatively, the "competition" effect which produces the difference in RFP protein expression levels could be present at both the transcription and translation levels, and could also explain the discrepancy in the RNA and protein data from the vcath and p10 samples.

The case for the large influence of RNA produced at earlier time points on protein production is further supported by several lines of evidence. While RFP RNA levels in the p10 samples increased to almost that of the gp64 sample levels at later times post- infection, protein expression does not show a correspondingly high level in p10 samples. This could be because the levels of RFP RNA started increasing earlier in the gp64 construct, than the p10 construct, and this drove early production of RFP protein in cells infected with the gp64 construct. The later start of RNA transcription in the p10 samples could have then had a much smaller impact on RFP protein production in cells. In addition, the basic samples, which showed highest early GFP

RNA and protein levels, showed the lowest levels of RFP protein and RNA. When compared to p10 samples, the levels of RFP RNA produced from the basic construct was much lower than that produced from the p10 construct. This is in spite of the fact that the levels of p10 GFP RNA were much higher than basic GFP RNA during later time points. Because the levels of GFP RNA were lower in the p10 construct compared to the basic construct during earlier time points, this could indicate that the levels of GFP RNA at early time points influenced the lower production of RFP RNA from the basic construct, which indicates that competition is especially influenced by the RNA production at early time points.

The normalized GFP and RFP values were used to generate a plot of the ratio of GFP to RFP RNA over time, similar to that in Chapter 3 (George et al., 2015), which is reproduced in Figure 4-3 B. While the general shape of the RNA plot seems to be the same as that of the protein, it seems to be shifted to earlier times post infection relative to the protein expression ratios. This could be due t o the time required to produce protein from RNA transcripts, as well as due to the time required for the produced polypeptide chain to mature into a fluorescent protein. This should be taken into account when designing systems for expressing proteins in predetermined ratios, where the amount of time needed to produce different proteins may vary, and so the protein expression profiles would be different at different times post infection.

Interestingly, the ratios of GFP protein to GFP RNA are similar at most time points except for the two highest GFP expressing p10 and basic constructs, where the ratios are much larger (Figure 4-3). The ie1 construct shows the next highest values, followed by the low GFP producer vcath and gp64 constructs. This could indicate that there is an “amplification” effect during protein production, where an increase in transcription results in a much higher increase in translation. The differences in ratios were not as obvious for RFP production, with only the vcath

construct having a markedly higher ratio than the other constructs. Due to the ratios not changing significantly over time after the infection event, it is thought that the competition effect is mainly determined at the transcription step.

The RNA tracking data for the dual protein expressing constructs suffers in that there is high variability in the data. This is due to the systemic variability that is present when samples are run through the real time PCR process. The cell culture experimental setup involved infecting and analyzing three insect cell flasks for each of the five baculovirus constructs used in this work, for a total of fifteen flasks. The samples from the first flask of each set of triplicates were regarded as one set, the second flask of each set of triplicates as the second set, and the third flask of each of the triplicates as the third set. The real time PCR setup used in this work involved running one set of samples (for example samples from 16 – 96 hours of the first flask from a set of triplicates) of each of the constructs, on one qPCR plate. Therefore, complete characterization of each of the fluorescent protein RNA quantities required three plates (one for each set of samples). While the differences between constructs on each of the plates were consistent, the absolute values of replicates (from triplicate flasks) varied from plate to plate. Due to this, the data has high apparent variability between replicates (Figure A-2), and this is a serious drawback to this study.

Chapter 5 : Formation of Dual Fluorescent Influenza Virus-Like Particles by Expression of Fluorescent Hemagglutinin and Matrix 1 Fusion Proteins in Insect Cells

This work has been submitted to the Journal of Biotechnology and is pending review.

5.1 Abstract

Cell culture-produced Influenza virus-like particles (VLPs) have been investigated extensively for use as vaccine candidates, and are in the process of being commercialized. However, much work still needs to be done on improving the VLP production process. This work reports on the generation of Influenza hemagglutinin and matrix 1 proteins that have been fused with fluorescent proteins, which then assemble into dual fluorescent VLPs. These fusion proteins enable tracking and monitoring of different stages in the VLP production process, from protein synthesis and localization within the cell, to the incorporation of component proteins into active VLPs, which can be detected using flow cytometry. It is hoped that this will provide a useful tool to study the VLP protein production process in cell culture, and help identify and remove the significant bottlenecks associated with high titer Influenza VLP production.

5.2 Keywords

Influenza virus-like particle

Hemagglutinin

Matrix 1

Fluorescent tagged proteins

Dual fluorescent virus-like particle

Baculovirus expression vector insect cell system BEVS

5.3 Introduction

Influenza A viruses are a significant cause of morbidity and mortality in the modern world. While vaccines have been shown to be effective in preventing or reducing the severity of the disease, the high rate of mutation and genetic re-assortment in these viruses requires the production of new vaccines every flu season. The two most common types of vaccines currently used contain either live attenuated Influenza virus or inactivated virions, and both are currently produced using embryonated chicken eggs. This is, however, a time and resource intensive process and is unsuitable for the production of vaccines against recent H5N1 viruses, which kill chicken eggs (Quan et al., 2007). In addition, an influenza pandemic may significantly affect the supply of eggs for vaccine production. Therefore, there has been a movement afoot in the past decade or so to move the production away from eggs and into cell culture. Today, Influenza vaccines based on the full virus are also made in MDCK cells, and more recently, seasonal flu vaccines based solely on the HA protein, are being made in insect cells by the Protein Sciences Corporation. The latter, and other vaccines based on virus-like particles (VLPs), i.e. incomplete and non-replicative virus particles, have the advantage of minimizing concerns related to the release of genes associated with virulent strains into the environment (Latham and Galarza, 2001). Significant process challenges still remain in the large scale cell culture-based production of VLPs for use as vaccines (Palomares and Ramírez, 2009). These challenges stem from the fact that VLPs are complex particles comprised of one or more proteins that have to be correctly assembled to form intact VLPs.

Influenza viruses are enveloped RNA viruses belonging to the Orthomyxoviridae family of viruses. Cell culture-propagated Influenza viruses are usually spherical between 80 and 120 nm in diameter (Lamb and Choppin, 1983). The Hemagglutinin (HA) glycoprotein, which exits

as a homotrimer, is embedded in the virus lipid coat. The portion of HA that protrudes to the environment gives rise, in part, to the antigenicity of the particle. HA mediates the binding of the Influenza virus to sialic acid residues on target cells, as well as membrane fusion between virus and cell (Wiley and Skehel, 1987). Another important component of the virus particle is the matrix M1 protein, which binds viral RNA and, along with HA and neuraminidase (NA), make up the structural component of the virus. In the insect cell system, Influenza VLPs have been produced with four proteins (HA, NA, and M1 and matrix 2 (M2)) (Latham and Galarza, 2001), three proteins (HA, NA, and M1) (Bright et al., 2007; Pushko et al., 2005; Pushko et al., 2007; Thompson et al., 2015), and only the HA and M1 proteins (Quan et al. 2007). The production of these proteins within insect cells result in the formation of particles resembling wild type Influenza virions, which range in size between 80 – 120 nm (Quan et al., 2007). Work on Influenza VLP production in the baculovirus insect cell system has led to the development of a series of pandemic and seasonal Influenza vaccines from Novavax, which are in Phase 1 and Phase 2 trials respectively (Novavax website, October, 2015). However, while the VLP production process in insect cells has been examined (Krammer et al., 2010; Thompson et al., 2015), there is still much work to be done on improving efficiency of the system.

A major impediment to improving the process is the difficulty in tracking the Influenza virus-like particles. Fluorescently tagged parvovirus B19 VLPs (Gilbert et al., 2004; Gilbert et al., 2005) and HIV (Gutiérrez-Granados et al., 2013) have been produced, and such a strategy has immense potential for characterizing and evaluating the entire manufacturing process. In this work, we aimed to implement such a strategy to create a particle that could aid in process development. More specifically, we report herein the creation of fluorescently tagged Influenza

virus HA and M1 proteins that form dual fluorescent functional VLPs, i.e. VLPs that maintain hemagglutination activity.

5.4 Materials and methods

5.4.1 Baculovirus Vector Construction

A baculovirus construct co-expressing Influenza hemagglutinin bound to enhanced green fluorescent protein (HA-GFP) and Influenza M1 attached to DsRed2 red fluorescent protein (M1-RFP) was generated for this work (Figure 5-1). The sequences for the *eGFP* and *DsRed2* genes, henceforth referred to as GFP and RFP respectively, were determined from plasmids pcDNA3-EGFP, which was a gift from Doug Golenbock (Addgene plasmid # 13031) and pCALNL-DsRed, which was a gift from Connie Cepko (Addgene plasmid # 13769 (Matsuda and Cepko, 2006)), and obtained from Addgene (Cambridge, MA, USA). The sequences for the *HA* and *M1* genes from Influenza A virus (strain A/Puerto Rico/8/1934 H1N1) were determined from GenBank (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda MD, USA). The sites for insertion of fluorescent genes into the Influenza genes were based on previous studies and were determined to be after the signal peptide at amino acids 1 – 17 for GFP into the Influenza *HA* gene (Li et al., 2005), and at the 3' end of the M1 gene, for the *RFP* gene linked to the M1 C terminus (Shishkov et al., 1999). The fluorescent genes were separated from the Influenza coding sequences using the flexible linker VDADS (Linhult et al., 2003). The expression cassette consisting of the *HA-GFP* gene in the opposite sense, under the control of the *p10* promoter from the plasmid pAcUW51 (BD Pharmingen, San Diego, CA, USA), and the *polh* promoter from the same plasmid upstream of the *M1-RFP* gene,

were synthesized and placed in a pMK vector (Life Technologies, Burlington, ON, Canada). The region of interest was then extracted, and 15 bp fragments were added to the ends of the sequence of interest by PCR using Phusion® High-Fidelity polymerase (Thermo Scientific, Waltham, MA, USA) using the primers: 5' – GGTAATGGTAGCGACCGGCGCTCAGCTGGAATT TCA GAT GCA TAT TCT GCA CTG CAA AG – 3' and 5'- CTA GCG CTT AAT AAA TGT ACT AAT AAC CGG ATC CCT ACA GGA ACA GGT GGT GGC GGC -3'. The underlined regions allow for integration of the extracted DNA fragment into a linearized baculovirus transfer vector. The plasmid pAcUW51 was linearized using the enzymes FastDigest™ BamH1 and EcoR1 (Thermo Scientific, Waltham, MA, USA). The expression cassette containing the Influenza genes and the linearized plasmid were ligated together using the In-Fusion PCR Cloning Kit (Clontech Laboratories Inc, Mountain View, CA, USA). The resulting plasmid contained the HA-GFP gene under control of a baculovirus p10 promoter and the M1-RFP gene under the control of a baculovirus polyhedron promoter (Figure 5-1). The baculovirus was generated from this vector using the BD BaculoGold™ Transfection Kit (BD Pharmingen, San Diego, CA, USA).

5-1: Position and orientation of gene sequences within pAcUW51 transfer vector. The eGFP coding region was inserted in between the N terminal influenza HA signal peptide, and the rest of the HA coding sequence. The DsRed2 coding sequence was added to the 3' end of the M1 gene

5.4.2 Cell Culture and Virus Production

Spodoptera frugiperda clonal isolate 9 (Sf9) cells (GIBCO, Carlsbad, CA) were maintained in capped glass Erlenmeyer flasks in Sf900III media (GIBCO) at a temperature of 27°C on an orbital shaker rotating at 130 rpm. Maintenance cell cultures were kept at a density between 0.5 and 4×10^6 cells/mL.

Baculovirus cultures were amplified by infecting Sf9 cells at a density of 1×10^6 cells/mL and allowing the infection to proceed until the viability dropped to 70–80%, after which cultures were harvested and centrifuged at 1000×g for 10 min in order to spin down cells and cell debris. The supernatant was removed and used as baculovirus stock for experiments. Baculovirus stocks were quantified by flow cytometry (Shen et al., 2002) and the end point dilution assay (King and Possee, 1992; Reed and Muench, 1938).

5.4.3 Native Polyacrylamide Gel Electrophoresis (PAGE)

Influenza protein detection was conducted by native PAGE using standard protocols. Briefly, samples were run on a 12% gel made with 29:1 polyacrylamide/bisacrylamide. Samples mixed with 5 × loading buffer (50% glycerol, 0.5% bromophenol blue, 0.5M Tris-HCl, pH 6.8), and the gel was run in running buffer (25 mM Tris, 192 mM glycine, pH 8.3) for 70 minutes at 200V. Gels were visualized using a blue-light transilluminator (Pearl Biotech, San Francisco, CA, USA) and a Smartview 310 gel imaging system fitted with a Canon PowerShot G16 camera (Discovery Scientific, Kelowna, BC, Canada). Cell lysate samples were prepared by resuspending pelleted cells in RIPA buffer (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate), 50 mM Tris, pH 8.0) supplemented with 1% n-dodecyl β-D-maltoside (DDM, Fisher Scientific, Toronto, ON, Canada), shaking samples on an orbital shaker at 4°C for 30 minutes, followed by centrifugation at 12,000 rpm for

10 minutes at 4°C. The supernatant was then separated and used for experiments. Samples of cells infected with viruses producing only GFP or RFP were treated with NP-40 buffer (150 mM sodium chloride, 1.0% NP-40, 50 mM Tris, pH 8.0), which lyses the cell and efficiently releases free GFP and RFP from cells. Infected cell culture supernatant was also concentrated 10× by ultrafiltration using an Amicon Ultra -15 10K centrifugal filter device (Merck Millipore, Carrigtwohill, Cork, Ireland). These were also treated with 1% DDM and used for experiments.

5.4.4 Ultracentrifugation

The purification of Influenza particles from culture supernatant was conducted by ultracentrifugation using an iodixanol gradient (Hutchinson and Fodor, 2014). Briefly, a 14 × 89 mm polyallomer centrifuge tube (Beckman Coulter, Mississauga, ON, Canada) was filled with 12 mL of culture supernatant, following which the tubes were spun in an Beckman Coulter SW 41Ti rotor (Beckman Coulter, Mississauga, ON, Canada) at 26,000 rpm (115,915 g) for 90 minutes. Following this, the supernatant was aspirated out, and the pellet was resuspended in 500 µL of NTC buffer (1 M NaCl, 0.2 M Tris-HCl pH 7.4, 50 mM $CaCl_2$). A density gradient was then prepared in a 14 × 89 mm polyallomer centrifuge tube with different densities of iodixanol OptiPrep™ Density Gradient Medium (Sigma Aldrich, St. Louis, MO, USA). These were 10, 15, 20, 25, 30, 35 and 40% iodixanol solutions prepared in NTC buffer, and 1.5 mL of each of these solutions was layered one at a time using a pipette. The resuspended virus pellet was then layered on top of the 10% gradient, and the tubes loaded into the SW 41Ti rotor and centrifuged for 35,000 rpm (209,000 g) for 150 minutes. Fractions were then collected by puncturing the side of the tube and analyzed by flow cytometry and transmission electron microscopy (TEM).

5.4.5 Electron Microscopy

Electron microscopy was conducted using a Philips CM10 transmission electron microscope

(TEM). Sample preparation was conducted by placing 5µl of undiluted sample onto parafilm and placing a 200 mesh carbon formvar coated copper grid (Ted Pella Inc, Redding, CA, USA) upside down onto the sample drop for 15 minutes. The grid was then placed into a drop of 2% formaldehyde in HEPES buffer for 10 minutes, following which the sample was placed into a drop of 3% aqueous phosphotungstic acid (PTA) stain pH balanced to pH 7.3 using an NaOH solution. After 45 seconds, the sample was removed from the drop, and PTA was wicked off with an absorbent wipe so as to leave only a thin film of stain on the grid. Sample grids were dried overnight prior to TEM imaging. Fully replicative Influenza A virus (H1N1 Puerto Rico/8/1934) produced in culture was a kind gift from the National Research Council of Canada (Montreal, QC, Canada) and was used as a control to compare with produced Influenza VLPs.

5.4.6 Confocal Microscopy

Confocal microscopy samples were prepared by first seeding 1×10^6 cells onto sterile glass cover slips (22 × 22 mm – No 1.5) placed inside a 35 mm tissue culture dish or in a 6-well plate. The cells were allowed to attach overnight and then infected with virus. The infection was allowed to progress for varying lengths of time. The sample preparation protocol involved aspirating media from the plate, and rinsing the coverslip 5 – 7 times with 1×PBS followed by aspiration of the spent PBS. The cells were then fixed with 4% formaldehyde in PBS (pH 7.4) for 15 minutes at room temperature, following which the sample was washed with PBS 5 – 7 times. A drop of ProLong® Gold Antifade Mountant (Life Technologies Inc., Burlington, ON, Canada) was placed on a 76.2 × 25.4 × 1 mm microscope slide, and the cover slip was placed upside down onto the drop of mountant. The mountant was then allowed to cure for 24 hours in the dark, following which the coverslip was sealed using paraffin wax and a cotton swab. The samples were then stored in the dark at room temperature for the short term till imaging. Confocal

microscopy was conducted using a Zeiss LSM 510 confocal microscope (Zeiss Canada, Toronto, ON, Canada).

5.4.7 Imaging Flow Cytometry Analysis (Cells)

Imaging flow cytometry was conducted on unfixed cells in suspension using an ImageStream^X Mark II (Amnis, Seattle, WA) imaging flow cytometer with 488 nm, 642 nm, and 785 nm lasers. Acquired flow cytometry files were analyzed, and images processed and exported using IDEAS software (Amnis, Seattle, WA).

5.4.8 Hemagglutination Assay

The presence of Influenza VLPs in samples was determined by the hemagglutination assay (Hirst, 1942), using well established protocols. Briefly, 50 µL of PBS was added to the wells of a round bottom 96-well plate, following which 50 µL of sample was added to the first well. Serial dilutions of sample were then conducted by mixing and transferring 50 µL of diluted sample sequentially to produce a row of wells with sequentially two-fold diluted samples. 50 µL of 0.5% chicken red blood cell suspension (Rockland Immunochemicals Inc. Limerick, PA, USA) in PBS was then added to each well, and the suspension allowed to sit at room temperature for an hour, following which results were read. Hemagglutination activity was noted by the lack of a red blood cell pellet at the bottom of wells. The highest dilution of samples which showed complete hemagglutination activity was noted and the reciprocal of the dilution factor was regarded as the number of Hemagglutination Units (HAU)/50 µL. This was then multiplied by 20 to derive the HAU/mL of sample.

5.4.9 Flow Cytometry Analysis (VLPs)

Flow cytometry was conducted on a BD FACS Calibur (BD Biosciences, San Jose, CA). The results were analyzed using the FlowJo software (Tree Star, Ashland, OR). Green fluorescence was detected by the FL1 detector (emission 530nm, bandpass 30 nm) and red fluorescence by the FL3 detector (emission 670 nm, longpass). Compensation was conducted to remove signal overflow from GFP fluorescence into the FL3 channel and was set at 11.5% of the FL2 signal based on the level of fluorescence detected when cells were infected with a monocistronic vector containing the GFP gene. Particle counts were obtained by running diluted samples through the cytometer and comparing particle counts with Flow-Set^{TM} fluorospheres (Beckman Coulter, Mervue, Galway, Ireland) of a known concentration. The baculovirus population was removed from VLP scatterplots by defining gates on forward scatter vs side scatter scatterplots of ultracentrifuged supernatants of cell cultures infected with baculovirus producing non-fused GFP and RFP (George et al., 2015), as shown in Figure 5-5. The points falling outside of this "baculovirus region" were counted as dual fluorescing VLPs.

5.5 Results and Discussion

5.5.1 HA and M1 Fluorescent Fusions

The Influenza *HA* and *M1* genes used in this work were synthesized using the sequence of the Influenza A PR/8/34 H1N1 virus strain. The virus hemagglutinin (HA) protein is a single pass type I membrane protein that is often localized to the host apical membrane in epithelial cells. The first 17 amino acid residues correspond to a signal peptide, following which is a 511 aa extracellular domain, followed by a helical 21 aa transmembrane domain and finally a 16 aa

cytoplasmic tail domain (Winter et al., 1981). An enhanced green fluorescent protein (eGFP, henceforth referred to as GFP) polypeptide was inserted after the HA signal peptide which extends from amino acids 1 - 16, as this has been shown to be amenable to accepting insertions (Li et al., 2005). The location of the signal peptide was determined from the UniProt database (accession number: P03437). Flexible linker regions were added to the N and C termini GFP peptide to allow the separate domains to fold independently. The fused protein is referred to in this work as HA-GFP.

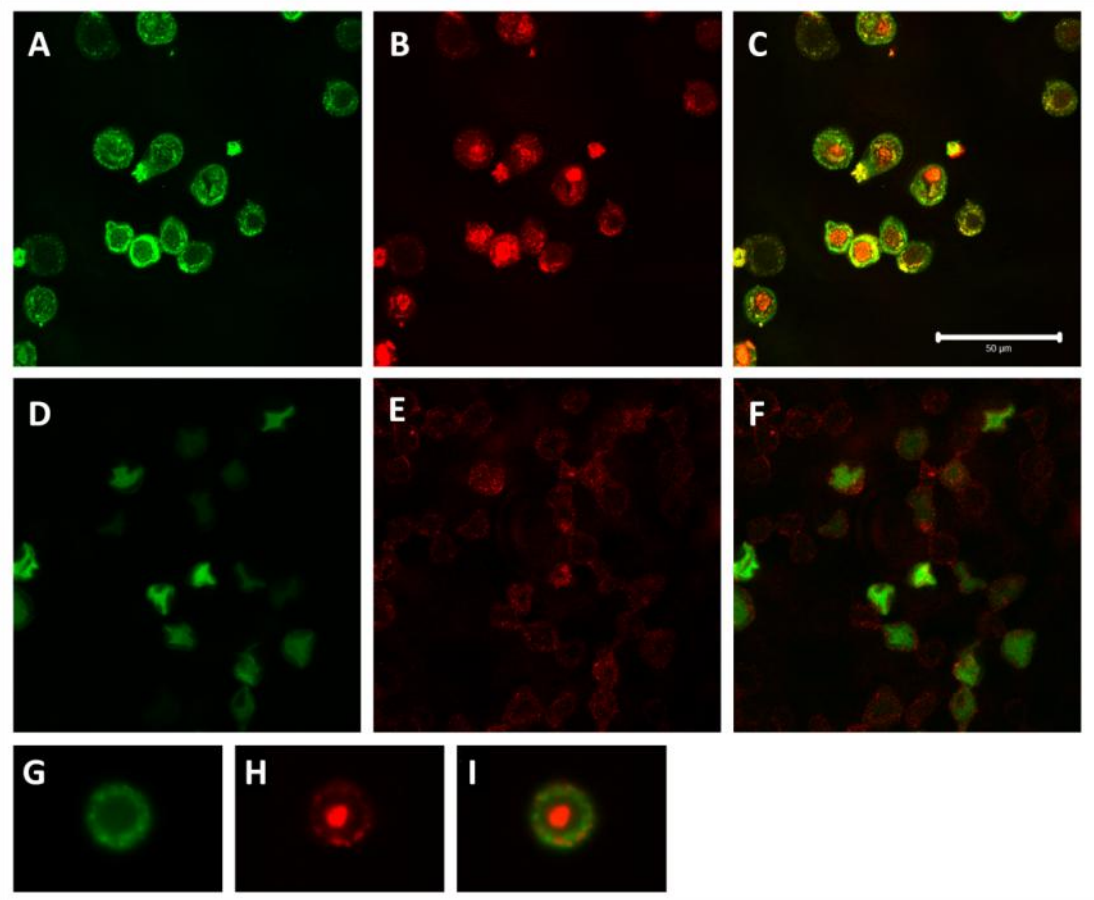
The M1 protein is a 252 aa protein that is arranged in a series of helical domains, with the N and C terminals facing towards the inside of the Influenza virion (Shishkov et al., 1999). In our work, a red fluorescent protein (DsRed2, henceforth referred to as RFP) polypeptide was added to the C terminus of the protein with flexible linker regions in between. This fusion protein is referred to as M1-RFP.

The production of fluorescent influenza proteins within Sf9 insect cells allowed for the easy visualization of influenza protein localization within the cell.

5.5.2 Protein Localization

Confocal microscopy was used to determine the localization of fluorescent Influenza HA and M1 proteins within Sf9 cells infected with the baculovirus expressing both virus proteins (Figure 5-2 A, B and C). It was found that HA-GFP localized mainly to the outer cell membrane, whereas the M1-RFP localized at the nucleus as well as parts of the membrane. This is different from when fluorescent proteins (eGFP, DsRed2) are expressed in insect cells (Sf9 or Hi5), where there is no distinct localization of the proteins and the entire cell either fluoresces red or green, even when the two proteins are co-expressed (George and Aucoin, 2015).The localization of HA-GFP

and M1-RFP was confirmed by using an imaging flow cytometer, where cells infected in suspension were passed through the cytometer (Figure 5-2 G-I), as opposed to confocal microscopy where the imaged cells were adhered to a glass slide, which indicates that the protein localization is independent of whether the cells are adhered to a surface, or in suspension. The localization of HA to the membrane was expected as the HA protein is an integral membrane bound protein which associates with lipid rafts (Scheiffele et al., 1997), which was confirmed by immunofluorescence staining (Wu et al., 2010).



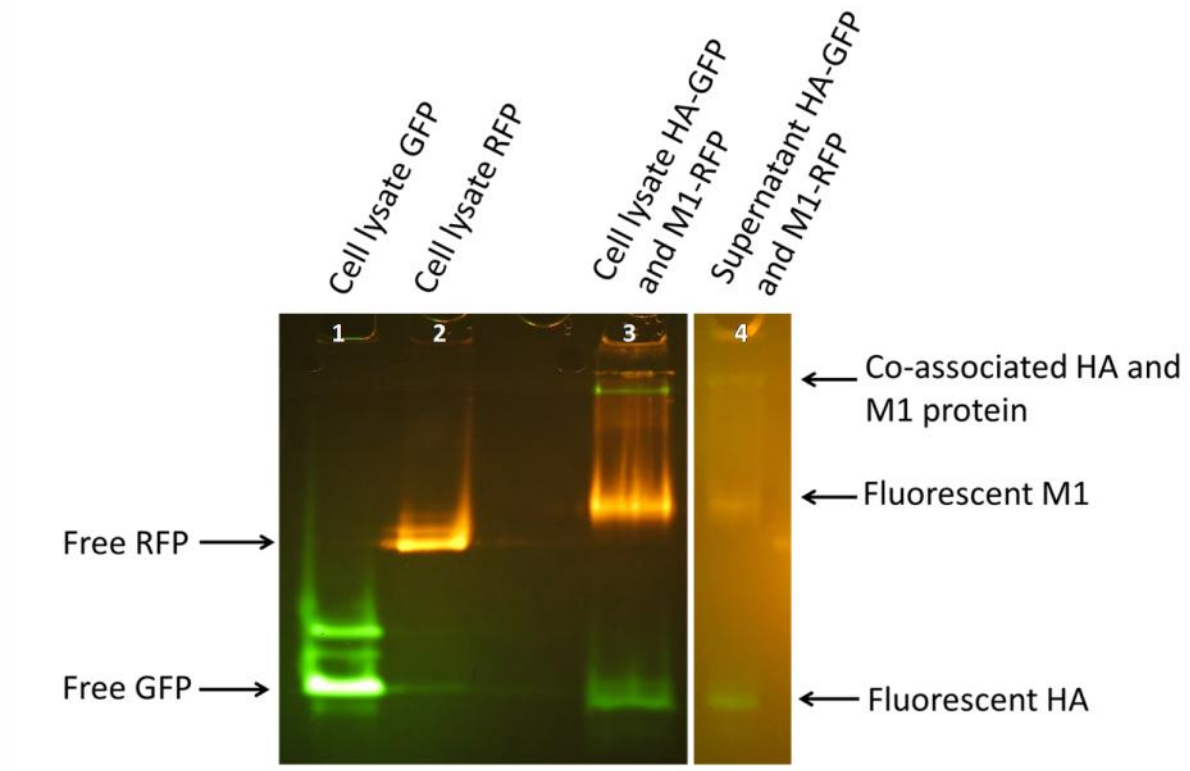
5-2: Confocal microscopy images of Sf9 cells infected with baculoviruses expressing HA-GFP (A) and M1-RFP (B) proteins. Panels D and E show the unfused GFP and RFP present throughout the Sf9 cells, without any specific localization. Imaging flow cytometry was conducted on cells in suspension, and used to show the presence of HA-GFP (G) and M1-RFP (H) in infected cells. Panels C and F are confocal overlay images of red and green fluorescence in the cells expressing Influenza fusion proteins, and unfused fluorescent proteins. Panel I is an imaging flow cytometry overlay of infected cells producing fluorescent Influenza fusion proteins. Figures 5-2 A, B and C were taken at the same plane in the cell sample, as were Figures 5-2 D, E and F. Similarly, Figures 5-2G, H and I were also taken at the same plane.

The membrane bound nature of the M1 protein has been well reported before, and could be due to either direct interaction of M1 with the membrane (Ruigrok et al., 2000), or through interactions with the cytoplasmic domain of membrane bound HA protein (Ali et al., 2000; Scheiffele et al., 1997; Zhang et al., 2000). The localization of M1 around the nucleus may be either due to the presence of a nuclear localization signal near the N terminal of the protein (Hui et al., 2003), or due to trafficking through the endoplasmic reticulum surrounding the nucleus after M1-RFP protein synthesis. The preferential localization of expressed M1 protein to the nucleus has also previously been observed in CV1 cells has been observed before, with a nuclear localization signal between amino acids 101 to 105 suspected as being the main driver of this behavior (Ye et al., 1995). This also suggests that the M1-RFP behaves similarly to unmodified M1 protein with respect to nuclear localization inside cells.

Detergent-solubilized virus-infected cell lysate, and ultrafiltration-concentrated cell culture supernatant, were run on a non-denaturing native PAGE gel (Figure 5-3) to observe the modified Influenza proteins produced in Sf9 cells. The use of a non-denaturing gel allowed for the use of the innate fluorescence of the modified proteins to monitor their migration through the gel. The HA-GFP and M1-RFP fusion proteins migrated differently when compared with unfused GFP and RFP. The HA-GFP band migrates faster than the free GFP band, an effect which could be due to some effect of tertiary structure of the HA-GFP or its surface charge. As this is a native PAGE gel, the migration distance is not indicative of relative protein size, which hinders the use of conventional protein ladders. The fusion protein lanes also showed additional bands resulting from protein not being able to migrate as far into the gel. Furthermore, these additional bands showed both green and red fluorescence, which could further point to a tight association between the two molecules where proteins stay associated even after cell lysis and

membrane solubilization. Similar banding patterns were also seen from cell culture supernatant samples (Figure 5-3, lane 4).The relative abundance of protein in each of the three bands in Lane 3 of the native PAGE gel is not indicative of the abundance of associated vs unassociated HA and M1 inside the cell, as there was a significant amount of protein that could not be dissociated from the membrane even after treatment with DDM, determined by the intense color associated with the membrane pellet after detergent treatment. Most of this un-dissociated protein is suspected to be membrane bound HA and associated M1.

5.5.3 PAGE gel Visualization of intracellular and supernatant fluorescent proteins



5-3: Native PAGE gel showing green and red fluorescence from cell lysate of cells producing GFP (Lane 1), RFP (Lane 2), HA-GFP and M1-RFP (Lane 3); and 10× ultrafiltration concentrated supernatant from cultures producing fused HA-GFP and M1-RFP (Lane 4).

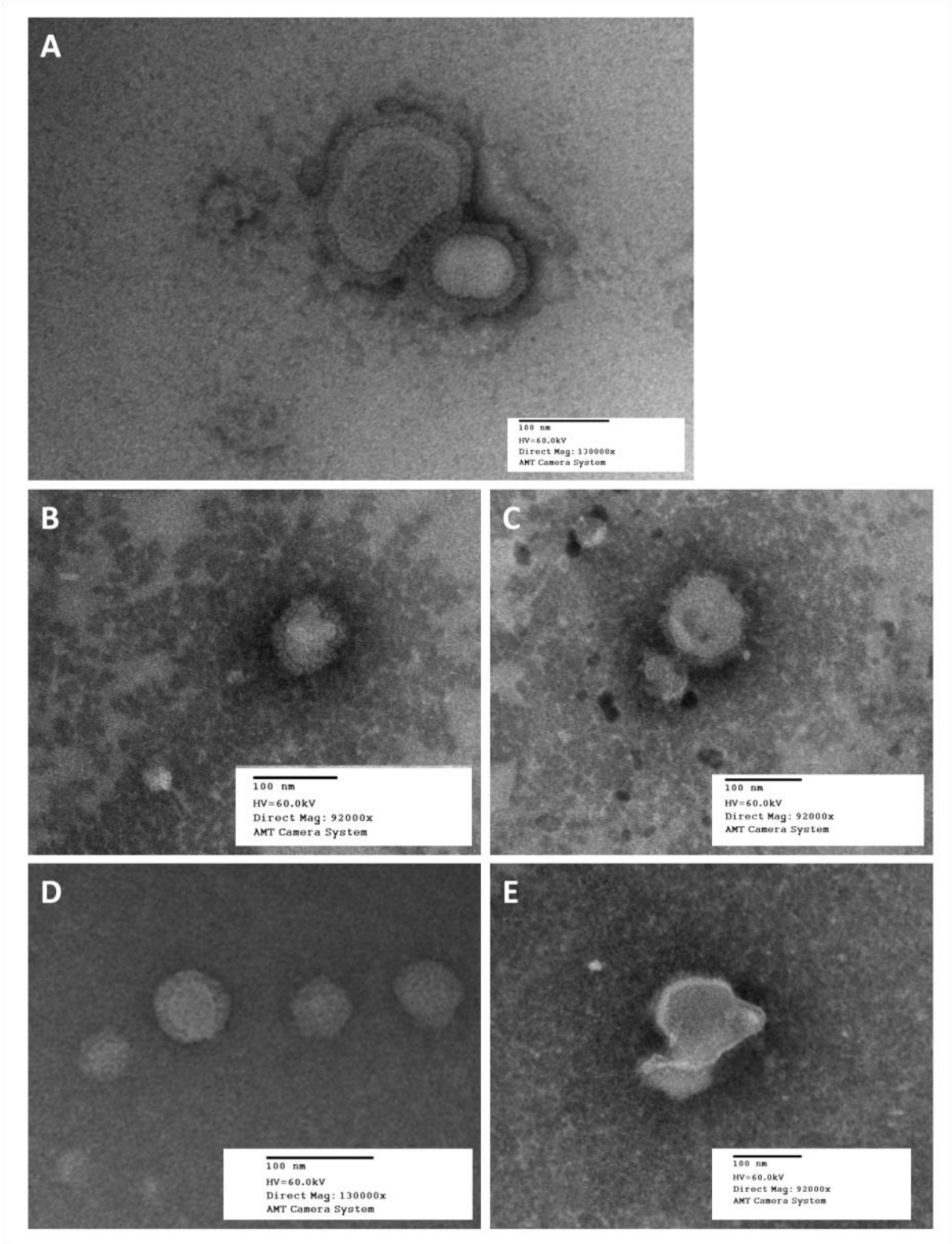
Further evidence of a tight association of M1-RFP and HA-GFP to the cell membrane came from cell lysis studies using either: a solution containing NP-40; or a solution containing Triton X-100

and DDM. Using NP-40, which is a relatively mild detergent, cells were lysed and the lysate was recovered through centrifugation. The majority of the fluorescence remained in the pellet fraction, indicating NP-40 was not able to release the fluorescent proteins effectively. Using the solution containing Triton X-100 and DDM, which are significantly more effective for releasing membrane bound proteins, a significant increase in fluorescence in the supernatant resulted following centrifugation. This is in marked contrast to when unfused GFP and RFP are expressed in cells, where the fluorescent proteins are easily solubilized by cell lysis with the mild NP-40 detergent, and the membrane pellet is completely decolorized.

Given that fluorescent proteins were also observed in the cell culture supernatant, it is plausible that some or all of this supernatant protein was from influenza VLPs containing fluorescent proteins which had been released into the supernatant. To examine the presence of VLPs in culture supernatant, further purification and concentration of the supernatant through an iodixanol gradient ultracentrifugation was performed.

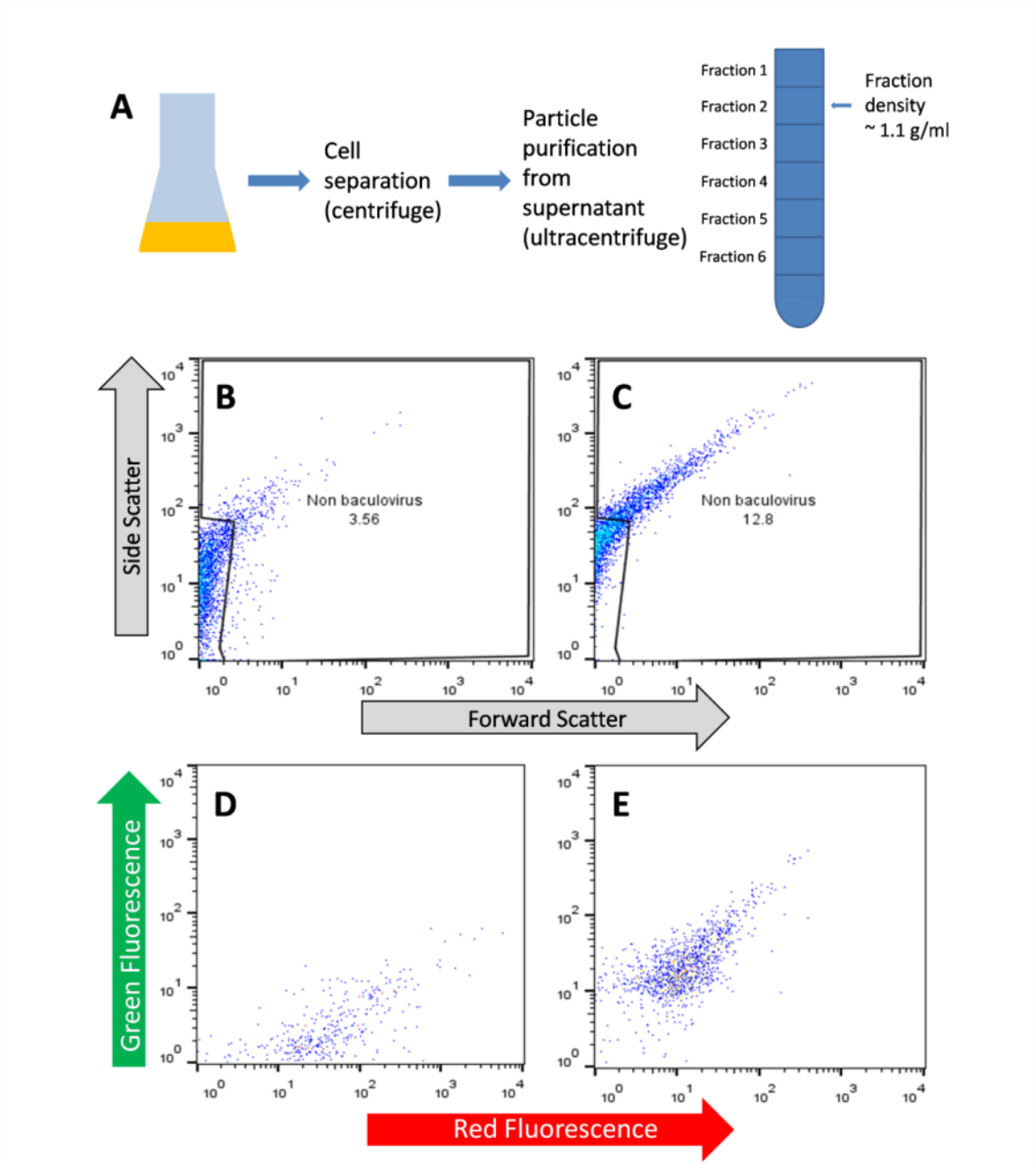
5.5.4 Extracellular Fusion Proteins and VLP formation

The presence of Influenza VLPs in infected cell supernatant was confirmed by TEM (Figure 5-4). These particles were identified by the presence of characteristic HA spike projections on their surface, as is present in the wild-type Influenza virus produced in cell culture (Figure 5-4A). Interestingly, the presence of a large GFP on the exterior of the VLP, as well as the RFP inside the VLP does not seem to affect particle morphology significantly.



5-4: Electron micrograph of wild type H1N1 PR/8/34 Influenza virus (A), and Sf9 produced VLPs (B, C, D and E).

5.5.5 Flow Cytometry



5-5: Flow cytometry scatterplot of ultracentrifuged supernatants produced from Sf9 cells. Panel A: Schematic of the processing steps used to obtain ultracentrifuge purified samples. Panels B and C: Forward scatter vs Sidescatter scatterplots between Influenza VLP producing constructs and non VLP producing constructs. B) Cultures infected with baculovirus producing unfused GFP and RFP.C) Culture infected with HA-GFP and M1-RFP producing baculovirus. Panels D and E: Scatterplot of green vs red fluorescence of events from the ungated region in Panels B and C, respectively.

Due to the fluorescent nature of the VLP component proteins, it was thought that these VLPs could be detected through the use of flow cytometry. Although flow cytometry is not designed to analyze viruses, it has been found to be useful in characterizing fluorescent and fluorescently labeled phage and viruses (Marie et al., 1999; Shen et al., 2002; Sokolenko et al., 2012). Given that we routinely quantify baculovirus by flow cytometry (Shen et al., 2002), we first ran samples of infected cell culture supernatant through an iodixanol density gradient ultracentrifugation step to isolate different fractions corresponding to different densities of liquid, with the rationale being that influenza VLPs would be present at the fraction density which corresponds to VLP density. These fractions were then run through a flow cytometer to examine if both green and red fluorescing particles could be detected and quantified. Based on forward and side-scatter, two distinct populations were observed for cultures producing GFP and RFP (Figure 5-5) and cultures producing HA-GFP and M1-RFP (Figure 5-5C). When gating out the particles observed in Figure 5-5B, and capturing only the events outside that gated area, there was a significant number of particles that were observed having high green and red fluorescence from cultures producing HA-GFP and M1-RFP (Figure 5-5E). Furthermore, although a few events from the cultures producing GFP and RFP (Figure 5-5B) fell outside of the gated area, these events did not have the same fluorescence profile (Figure 5-5D) as those from cultures producing HA-GFP and M1-RFP (Figure 5-5E).

The presence of fluorescent polypeptides attached to the Influenza proteins adds an increased layer of complexity in the assembly of the fusion proteins, and their incorporation into VLPs. In particular, the RFP used in this work (DsRed2) is an obligate tetramer (Bevis and Glick, 2002) that fluoresces in the red spectrum when assembled, though the dimeric form may also be fluorescent (Baird et al., 2000; Sacchetti et al., 2002). The intense DsRed2 signal seen in

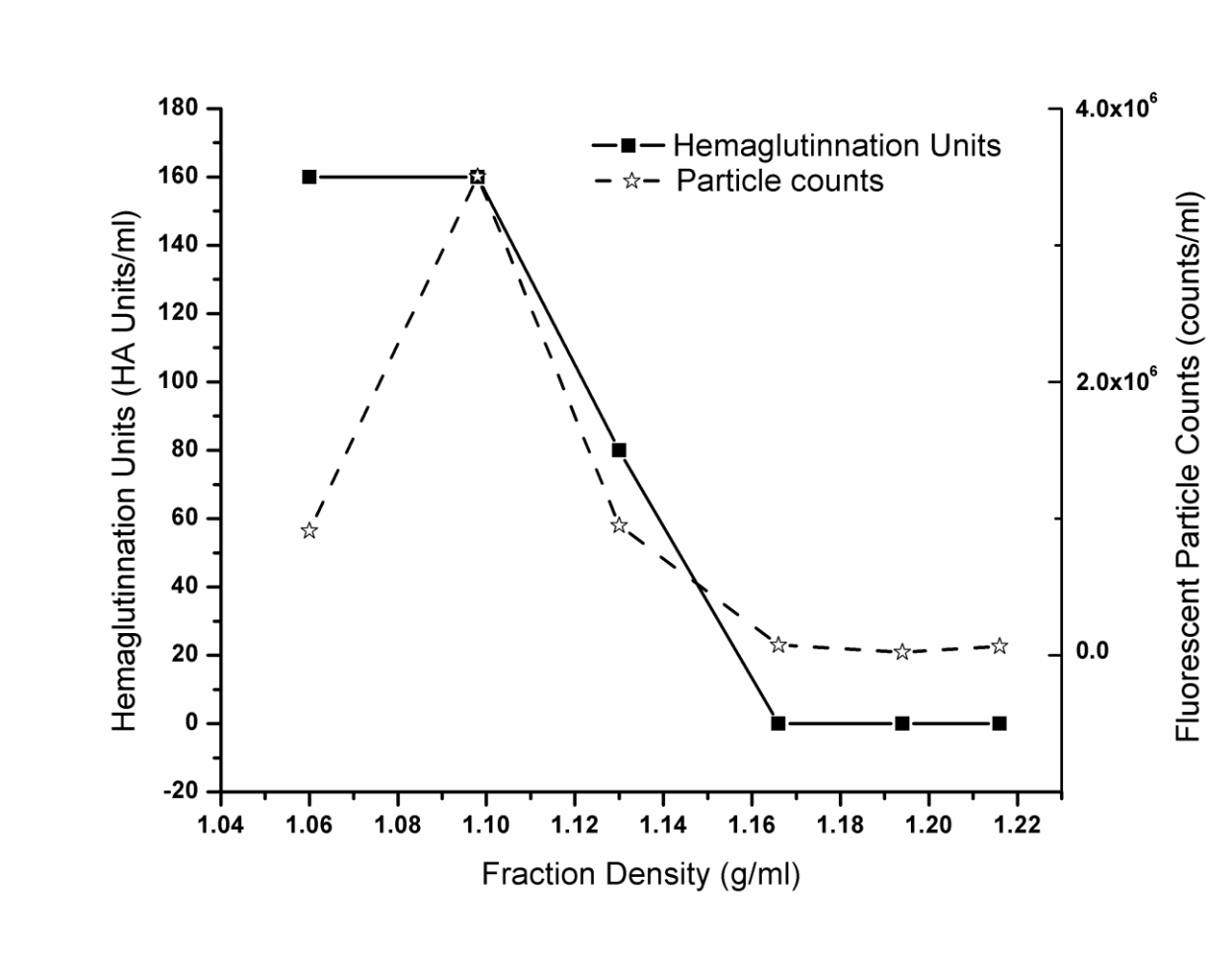
our experiments is an indication of complete and extensive maturation of the (DsRed2) protein within the system. The ability to detect red fluorescent particles by flow cytometry indicates that the tetramers remain assembled even within the virus-like particle. The free association of tetramers within the confined space of a virus-like particle may be facilitated by the flexible linker region separating the M1 from the RFP within the fusion protein. It is even possible that the collection of M1-RFP and its incorporation into VLPs is facilitated by the oligomerization of DsRed2.

Taken together, the detection of dual fluorescent events in iodixanol gradient ultracentrifugation fractions were indicators of VLP formation. The use of flow cytometry further allowed the quantification of events by comparing the particle counts in samples to a solution of calibration beads with known concentrations. Briefly, calibration bead solutions were run through the cytometer, and the time required to capture 10,000 events was noted. This was then compared to the time required to count 10,000 events from the ultracentrifugation fractions, and based on the concentration of the bead standard, the concentration of particles in the ultracentrifugation fractions could be determined. Therefore, this method could be developed further and used to monitor Influenza VLP production, and also accurately determine losses from downstream purification steps.

5.5.6 Hemagglutination Assay

Hemagglutination assays were conducted on ultracentrifuge purified culture supernatant to examine if the different ultracentrifugation fractions could cause hemagglutination, a test which has traditionally been used for the detection of influenza virus or VLPs in samples. The

hemagglutination activity followed the particle counts obtained from the flow cytometer of the various ultracentrifugation fractions (Figure 5-6).



5-6: Hemagglutination activity and particle counts of ultracentrifuge purified fractions of Sf9 culture supernatant. Reported particle counts have been modified to account for the concentration of particles from ultracentrifugation. Hemagglutination counts were obtained from 5× concentrated samples.

Hemagglutination activity was concentrated in the least dense ultracentrifugation fractions and was completely absent in the three densest fractions. The maximum flow cytometry counts of red and green fluorescent particles occurred in the second least dense fraction, and dropped down to negligible levels in the last three fractions. The discrepancy between the hemagglutination results and the flow cytometry counts, especially in the lightest fraction, could be due to a secondary population of particles that are either HA-only-containing VLPs which

would have been gated out during the flow cytometry analysis, or HA present on the surface of baculoviruses (Prabakaran et al., 2011; Yang et al., 2007). Even though the peak hemagglutination activity seen in Figure 5-6 was 160 HA units/mL (5× concentrated samples), an assay conducted on samples concentrated by ultrafiltration showed activity levels of ~320HA units/ml in a 10× concentrated supernatant samples, indicating that some hemagglutination activity was lost during the ultracentrifugation purification process. Hemagglutination activities obtained from supernatants were comparable to previous values (335 HA units/ml in iodixanol purified samples, and 32.3 HA units/ml in sucrose cushion purified samples, both expressed as HA units in non-concentrated samples) obtained from VLP production in Sf9 cells (Thompson et al., 2015). However, the VLP counts of ~4×10^6 VLPs/mL in non-concentrated supernatant obtained from flow cytometry in this work is significantly lower than titers of ~10^9 VLP/mL reported by Thompson et al. (Thompson et al., 2015). This could be due to several factors; however, one of the reasons may be because flow cytometry only captures red and green fluorescent VLPs, while electron microscopy-based methods may be capturing VLP particles that would not show up as dual fluorescent.

5.6 Conclusions

In our work, we have produced modified Influenza virus-like particles using only two Influenza proteins: the hemagglutinin (HA) protein embedded in the outer VLP membrane, and the matrix (M1) protein, which is enclosed by the membrane. These proteins were modified with fluorescent proteins to enable easy tracking of the resulting VLPs during their production: from individual protein synthesis within the cell, to tracking VLPs in supernatant. It is hoped that this

will provide a useful tool to simplify the process of examining Influenza VLP production in insect cells, as well as in other cell types.

Preliminary analysis of the VLP production process from these experiments indicates that only a fraction of the produced protein is excreted to the supernatant in the form of VLPs. Therefore, much work needs to be done to improve the assembly of protein into functional VLP product. This could involve placing both genes under the control of powerful early promoters to drive rapid protein export during the early stages of infection when the cell protein export mechanism is less compromised.

Chapter 6 : Controlling Expression of Influenza Proteins in Sf9 and High Five Cells Using Alternative Baculovirus Promoters to Manipulate VLP Composition

This manuscript is to be submitted shortly to a scientific journal.

6.1 Abstract

The increasing use of the Baculovirus Expression Vector System (BEVS) for protein production has given great incentive to improve its utility. In particular, the development of tools to provide greater control over the intracellular protein production process could be of use in producing complex biologics such as influenza VLPs. In this work we report the use of various promoter combinations to control expression of fluorescent influenza proteins in a baculovirus-insect cell coexpression system. The green fluorescent hemagglutinin (HA-GFP) and red fluorescent matrix 1 (M1-RFP) were produced at different levels in Sf9 and High Five™ cell lines using baculovirus vectors containing the two genes under the control of three promoter combinations. The level of expression of these proteins was shown to vary widely between constructs, as well as between cell lines. In addition, the level of expression of the HA-GFP and M1-RFP was shown to affect the composition of VLP particles produced in culture supernatant.

6.2 Introduction

The baculovirus insect cell system has been used extensively to produce a wide variety of protein products, especially complex multiprotein products such as virus-like particles (Sokolenko et al, 2012). However, the systematic study of protein production within the insect cell system is a more recent field of interest, and to date there have only been a few studies examining the effect of using alternative promoters to control the level or timing of expression of individual proteins and improve the yield of the final multiprotein product. In insect cells, alternative protein promoters have been used to increase yields of influenza VLPs (Quan et al., 2007), simian immunodeficiency VLPs (Kang and Compans, 2003; Yamshchikov et al., 1995), human immunodeficiency VLPs (Sailaja et al., 2007), and ebola VLPs (Sun et al., 2009; Ye et al., 2006). Most of these studies use the hybrid late/very late *Pcap/polh* to drive the expression of one or more VLP components, which allows for earlier expression of component proteins. Yamschikov et al (1995) directly compared the use of the *Pcap/polh* promoter to the very late *polh* promoter for improving the production of SIV envelope protein and discovered that the hybrid promoter increased the expression of SIV envelope protein and consequently SIV VLPs, when compared to the use of the purely "very late" *polh* promoter.

Recently, a systematic examination of the production of two fluorescent proteins following infection of Sf9 cells with a polycistronic virus was conducted to better assess the ability to control expression by manipulating baculovirus promoter sequences (George et al., 2015). The study revealed that protein expression levels and timing could be reliably controlled using different promoters, and that it was possible to set up expression schemes where a defined ratio of foreign proteins could be obtained. It was also found that multiple protein production within the same cell resulted in non-trivial competition effects, where the expression level of one

protein influenced the expression of other proteins. However, George et al. (2015) examined a simple co-expression system involving non-interacting fluorescent proteins, which, when produced in insect cells, did not localize to any particular region of the cell. For the concepts explored in that work to be of general use, the demonstration of promoter control and the interplay between protein expression levels at different points in time requires a more complex system. To this end, this work studies the production of influenza VLPs from the co-expression of influenza hemagglutinin (HA) and Matrix 1 (M1) fluorescent fusions in insect cells via infection with polycistronic baculovirus vectors.

Several studies have successfully produced influenza VLPs using two, three or four of the influenza structural proteins (hemagglutinin (HA), neuraminidase (NA), and matrix 1 and 2 (M1 and M2)), and have evaluated their efficacy as vaccines in animal models (Bright et al., 2007; Galarza et al., 2005; Kang et al., 2009; Pushko et al., 2005; Quan et al., 2007; Wen et al., 2009). Novavax have influenza VLP candidate vaccines that are in various stages of clinical trials. Despite the number of studies and advances in the area, there have been comparatively few studies on process considerations while producing the virus-like particles in insect cell culture (Krammer et al., 2010; Thompson et al., 2015).

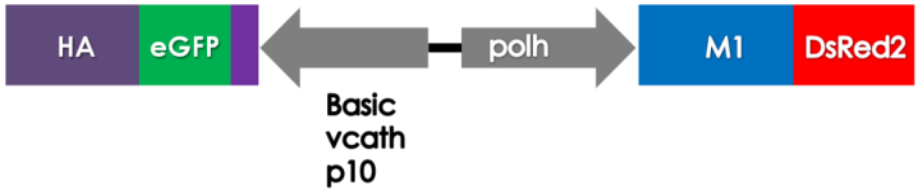
The production of influenza vaccines in insect cell culture has become an accepted alternative to production in embryonated chicken eggs, with Protein Sciences Corporation producing Flublok, which is composed of recombinant hemagglutinin antigens. Flublok is the only egg-free seasonal influenza vaccine available on the market. The insect cell system has also been used to produce several other vaccines including Cervarix® (GSK), with several other candidates undergoing clinical trials (Cox, 2012), and this provides significant impetus to continue research to improve the system.

The work presented here is a natural extension of the study on promoter control (George et al., 2015) and production of fluorescent influenza-like particles (Chapter 5). Using the genes for HA-GFP and M1-RFP (Chapter 5), two new polycistronic baculovirus vectors were created to have the HA-GFP downstream of either a basic or *vcath* promoter, and the M1-RFP downstream of the *polh* promoter. Together with the original construct (p10 HA-GFP, polhM1-RFP; (Chapter 5)), these three viruses, herein referred to as the basic, vcath, and p10 viruses respectively, were used to infect two different cell lines – Sf9 and High Five^{TM}. We show that similar to our previous study, protein production can be modulated in insect cells in a reproducible and predictable manner based on the choice of promoter sequence upstream of the gene of interest (despite different sizes and localizations); and competition effects observed with the simple fluorescent protein system are also apparent in this fluorescent influenza protein system (high levels of expression of one protein will influence the expression of a second protein being produced). We also show that the levels of individual protein production affect the composition of the final influenza VLP particles that buds out of the cell. Infecting the two cell lines with the same baculoviruses revealed that in general, while High Five^{TM} cell line produced far more protein than the Sf9 cell line, Sf9 cells produced more VLPs.

6.3 Materials and Methods

6.3.1 Baculovirus Vector Construction

Three baculovirus vectors co-expressing influenza hemagglutinin bound to enhanced green fluorescent protein (HA-GFP) and influenza M1 attached to DsRed2 red fluorescent protein (M1-RFP) were used in this work. The construction of a plasmid containing the HA-GFP gene under control of a baculovirus *p10* promoter and the M1-RFP gene under the control of a baculovirus *polh* promoter has been described earlier (Chapter 5). Two new constructs, prepared for this work, replaced the *p10* promoter with *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) *basic* and *vcath* promoters (named basic and vcath constructs) using overlap extension PCR (Bryksin and Matsumura, 2010) to produce a total of three constructs (Figure 6-1). The primers used to extract the promoters and to replace the p10 promoter are found in Table 6-1. Baculovirus vectors were generated from these constructed plasmid vectors using the BD BaculoGold™ Transfection Kit (BD Pharmingen, San Diego, CA, USA).



6-1: Schematic of expression cassette in pAcUW51 coding for the HA-GFP and M1-RFP fusion proteins

6-1: Primers for replacing p10 promoter (the underlined regions correspond to areas around the p10 promoter on the pAcUW51 vector where the new promoters integrated)

Promoter	Direction	Sequence
Basic	Forward	5'-CAACAAGGGGGACTATGAAATTATGCATTTGAGGATGCCGTTTTGCGACGATGCAG- 3'
	Reverse	5'– CTGGTAATGGTAGCGACCGGCGCTCAGCTGGAATTCGTTTAAATTGTGTAATTTA TGTAGCTGTAATT – 3'
Vcath	Forward	5' –GGACTATGAAATTATGCATTTGAGGATGAATTTATCTTAATTTTAAGTTGAATTCCAGCT – 3'
	Reverse	5' – GGTAGCGACCGGCGCTCAGCTGGAATTCAACTTAAAATTAAGATAAATTCATCCTC –3'

6.3.2 Cell Culture and Virus Production

Spodoptera frugiperda clonal isolate 9 (Sf9) cells (GIBCO, Carlsbad, CA) were maintained in capped glass Erlenmeyer flasks in Sf900III media (GIBCO) at a temperature of 27°C on an orbital shaker rotating at 130 rpm. High Five™ cells (Thermo Fisher Scientific, Burlington, ON, Canada) were maintained in a similar fashion in Express Five® Serum Free media (Thermo Fisher Scientific, Burlington, ON, Canada) supplemented with 18 mM of glutamine. Maintenance cell cultures were kept at a density between 0.5 and 4×10^6 cells/mL.

Baculovirus stocks were generated as described previously (Chapter 5). Briefly, baculoviruses were amplified in Sf9 cells by infecting cultures with a low MOI (estimated to be 0.1) and recovering the supernatant when culture viability was between 70 – 80%. All virus used in this work was P2 virus. Titers were quantified by the end point dilution assay (King and Possee, 1992; Reed and Muench, 1938). All VLP production experiments were conducted by infecting cell cultures at 1×10^6 cells/mL with various viruses at an MOI of 5.

6.3.3 Confocal Microscopy

Confocal microscopy samples were prepared by seeding 1×10^6 cells onto sterile glass cover slips (22 × 22 mm – No 1.5) placed inside a 35 mm tissue culture dish. The cells were allowed to

attach overnight and were then infected with virus for 48 hours. The coverslip was then rinsed 5 times with 1×PBS (pH 7.4) and then fixed with 4% formaldehyde in PBS (pH 7.4) for 15 minutes at room temperature, following which, the sample was rinsed again 5 times with PBS. The coverslip was then placed upside down on a drop of ProLong® Gold Antifade Mountant (Life Technologies Inc., Burlington, ON, Canada) on a 1 mm thick microscope slide, and the mountant was allowed to cure for 24 hours in the dark. Confocal microscopy was conducted on the cured sample using a Zeiss LSM 510 confocal microscope (Zeiss Canada, Toronto, ON, Canada).

6.3.4 Ultracentrifugation

The purification of influenza particles from culture supernatant was conducted by ultracentrifugation using an iodixanol gradient (Hutchinson and Fodor, 2014). Briefly, VLPs and baculoviruses were pelleted from 12 mL supernatant samples by ultracentrifugation at 26,000 rpm (115,915 g) for 90 minutes using a Beckman Coulter SW 41Ti rotor (Beckman Coulter, Mississauga, ON, Canada). The pellets were then resuspended in 500 uL of NTC buffer (1 M NaCl, 0.2 M Tris-HCl pH 7.4, 50 mM $CaCl_2$). An iodixanol density gradient was constructed with 10, 15, 20, 25, 30, 35 and 40% solutions of iodixanol OptiPrep™ Density Gradient Medium (Sigma Aldrich, St. Louis, MO, USA), and the resuspended virus pellet was layered on top of the 10% solution. These tubes were then centrifuged at 35,000 rpm (209,000 g) for 150 minutes in the same SW 41Ti rotor after which the different iodixanol gradient fractions were collected and analyzed using flow cytometry and transmission electron microscopy for the presence of virus like particles.

6.3.5 Transmission Electron Microscopy (TEM)

Electron microscopy was conducted on concentrated supernatant samples to detect the presence of influenza VLPs. Sample grids were prepared by placing a 200 mesh carbon formvar coated copper grid (Ted Pella Inc, Redding, CA, USA) onto a sample droplet for 15 minutes. The grid was then placed in 2% formaldehyde dissolved in PBS to fix the sample, after which it was stained in a drop of 3% aqueous phosphotungstic acid (PTA) stain pH balanced to pH 7.3 for 45 seconds. After this, excess stain was wicked off and samples allowed to dry overnight. The grids were imaged using a Philips CM10 transmission electron microscope.

6.3.6 Hemagglutination Assay

The presence of influenza VLPs in samples was determined by the hemagglutination assay, using a well-established protocol (Eisfeld et al., 2014). Briefly, serial dilutions of concentrated virus sample were added to chicken blood cells in round bottom 96 well plates and the suspension allowed to sit undisturbed at room temperature for an hour. The highest dilution of samples that showed complete hemagglutination activity was noted and the reciprocal of the dilution factor was regarded as the number of Hemagglutination Units (HAU)/50 ul. This was then multiplied by 20 to derive the HAU/ 1ml of sample.

6.3.7 Flow Cytometry Analysis of cells and purified culture supernatant

Cell fluorescence was obtained by fixing cells in formaldehyde solution and then running them through a BD FACS Calibur (BDBiosciences, San Jose, CA) flow cytometer equipped with a 488 nm laser. Green fluorescence was detected by 530 mm filter with a bandpass of 30nm, and red fluorescence was detected by a 670 nm longpass filter. Compensation was used to remove the GFP signal from the red fluorescence channel, and determined using cells infected with monocistronic vectors encoding for GFP.

Fluorescent influenza virus-like particles were detected by flow cytometry as detailed previously (Chapter 5), and using the same filters used for detecting cell fluorescence. Briefly, the regions on a scatterplot of forward scatter (particle size) and side scatter (particle complexity) corresponding to particles was determined using a control culture infected with a construct that did not produce VLPs. This region was then excluded out of scatterplots of VLP containing supernatant concentrates, and the green and red fluorescence of the resulting populations. Particle counts were obtained by running ultracentrifugation purified supernatant samples and comparing these to known particle counts from a calibrated control (Flow-Set^{TM}fluorospheres, Beckman Coulter, Mervue, Galway, Ireland).

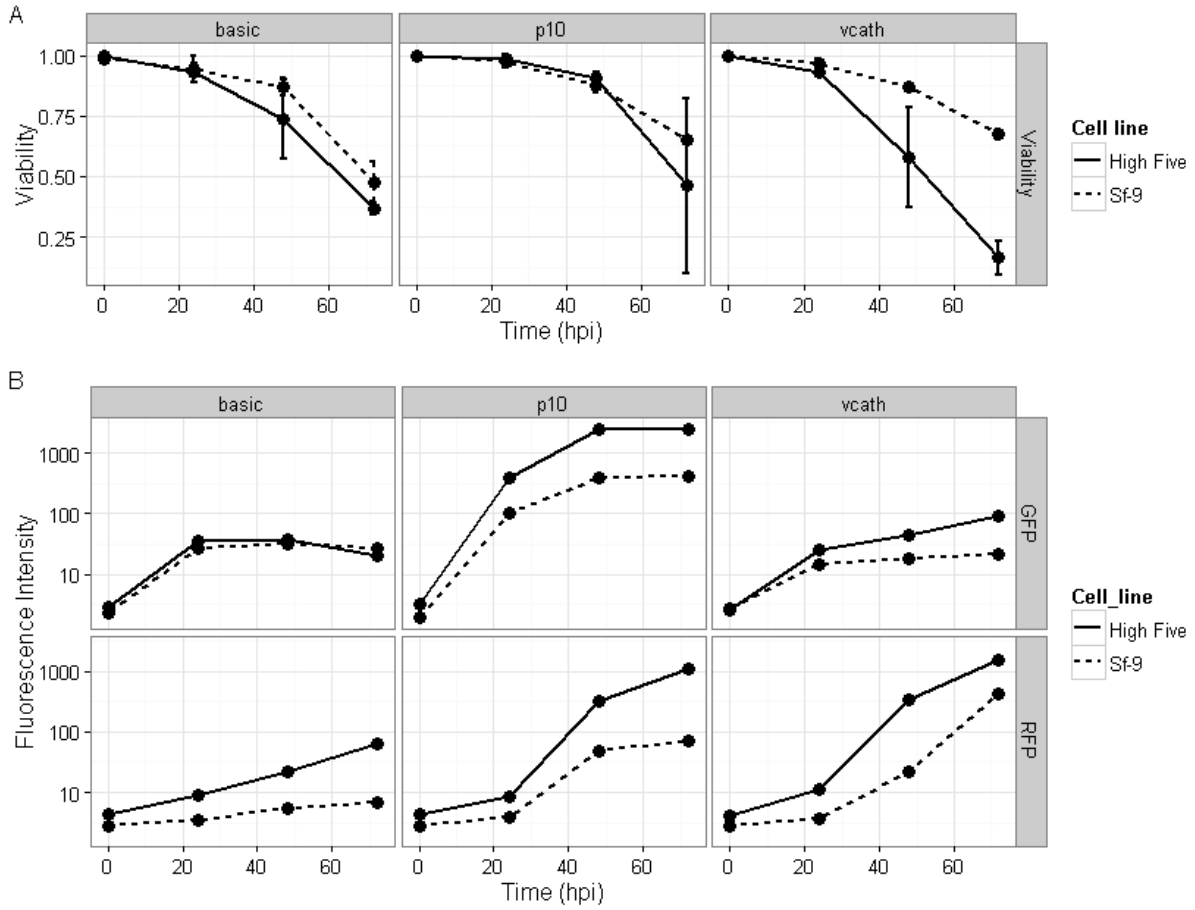
6.4 Results

6.4.1 Tracking Fluorescent Proteins in Cells

Flow cytometry was used to monitor fluorescent viral protein production in infected Sf9 and High Five^{TM} cells (Figure 6-2). The two cell types were infected with the basic, vcath and p10bicistronic baculovirus vectors.

In general, High Five^{TM} cells expressed higher amounts of HA-GFP and M1-RFP, except in the case of the basic construct, where the Sf9 and High Five^{TM} cells produced the same amount of HA-GFP. The results also show that higher production of one protein generally corresponded to a lower level of production of the other protein, which is consistent with previous findings in a simple two reporter protein system (George et al., 2015). This can be seen especially well in the vcath and p10 constructs where the vcath construct produced significantly

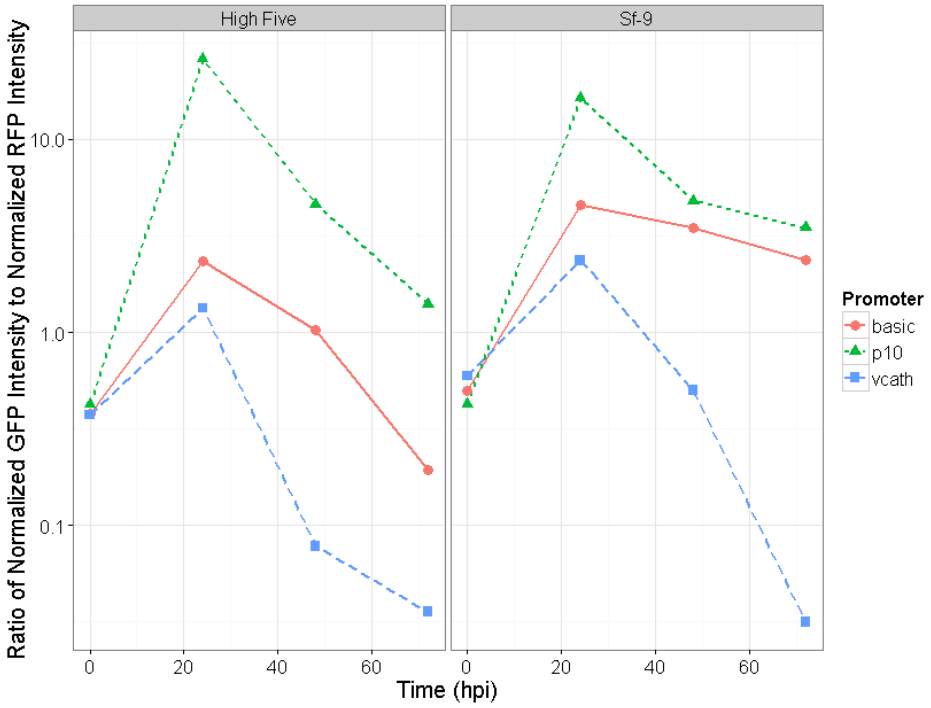
lower levels of HA-GFP, but much higher levels of M1-RFP than the p10 construct, even though the M1-RFP gene was under the control of the polh promoter in both constructs (Figure 6-2).



6-2: Protein expression from insect cell and culture viability over time: A) Viability of cells over the course of infection. B) Green (eGFP), and red (DsRed2) fluorescence values of Sf9 and High Five™ cells infected with the basic, vcath and p10 baculovirus constructs. Both Sf9 and High Five™ cells were infected at the: same density (1×106 cells/ml) and with the same numbers of virus. Error bars are small and represent the standard deviation of duplicate flasks.

Green and red fluorescence observed by flow cytometry was used to generate normalized expression ratios of green and red fluorescence in Sf9 and High Five™ cells over time after infection with the different baculovirus constructs (Figure 6-3). Data normalization was conducted by dividing fluorescence at each time point by the maximum green or red fluorescence in that dataset, following which the ratio of the normalized data was plotted. While not indicative of true protein expression ratios, the figure illustrates that while the basic and p10

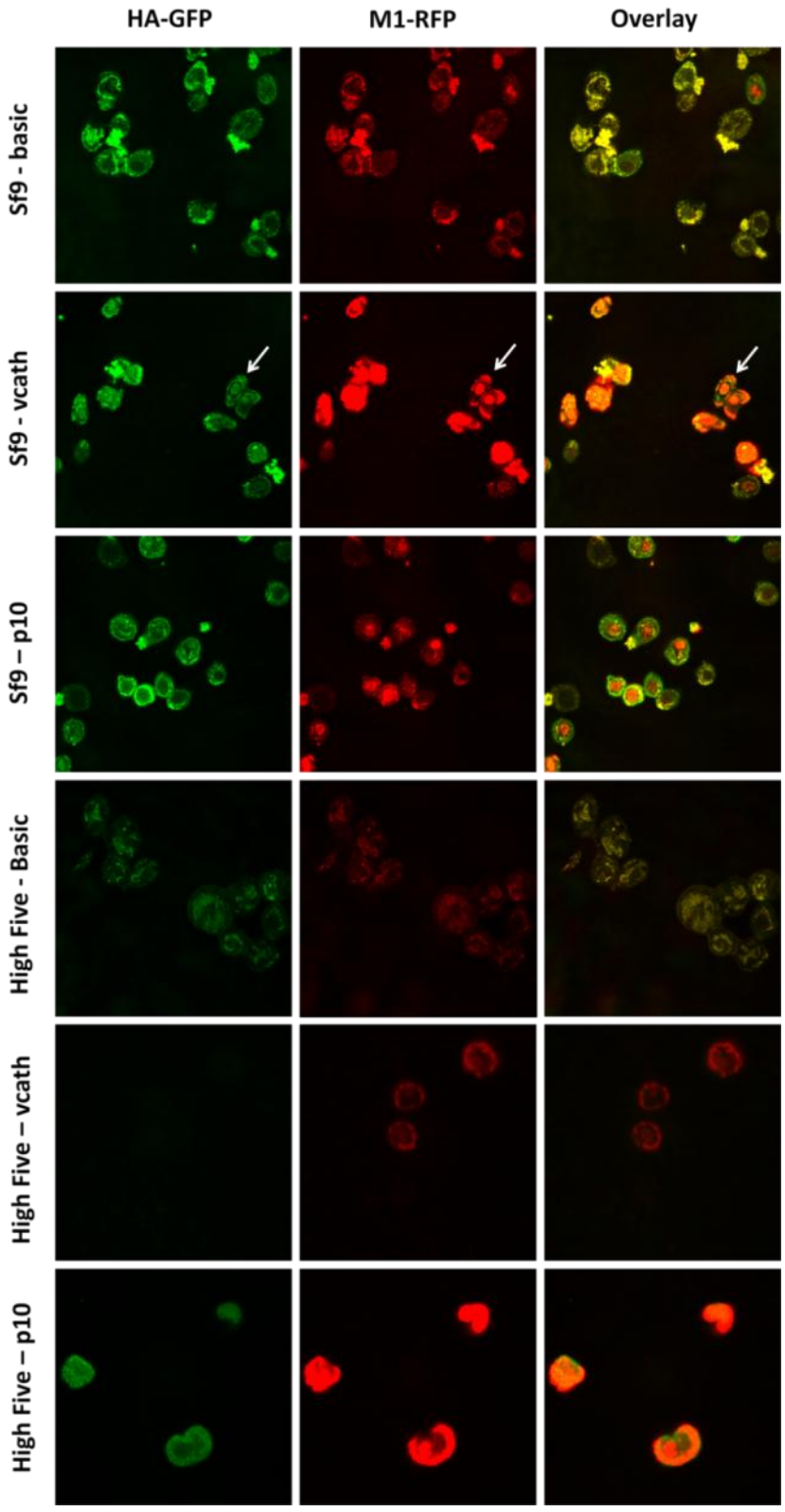
constructs produce large amounts of HA-GFP earlier in the infection cycle compared to M1-RFP, in the vcath construct HA-GFP is expressed at much lower levels than M1-RFP at all times post infection, and in both cell types. In addition, while similar expression ratios are seen from both High Five™ and Sf9 cells from the p10 and vcath constructs, the basic construct drives higher relative expression of HA-GFP as compared to M1-RFP in the Sf9 cells, as compared to the High Five™ cells.



6-3: Ratio of green and red fluorescence post-infection for different promoter combinations in Sf9 and High Five™ cells

The observations made by flow cytometry were verified by confocal microscopy (Figure 6-4), where the images show clearly the relative differences between the different constructs. The localization of HA-GFP to the cell membrane can be seen as a result of infection by all constructs, particularly in the Sf9 cells. M1-RFP protein, however, is present at both the cell membrane and the nucleus. Overlay images of green and red fluorescence in Sf9 cells indicate a high degree of co-localization of HA-GFP and M1-RFP particularly at the cell membrane. A

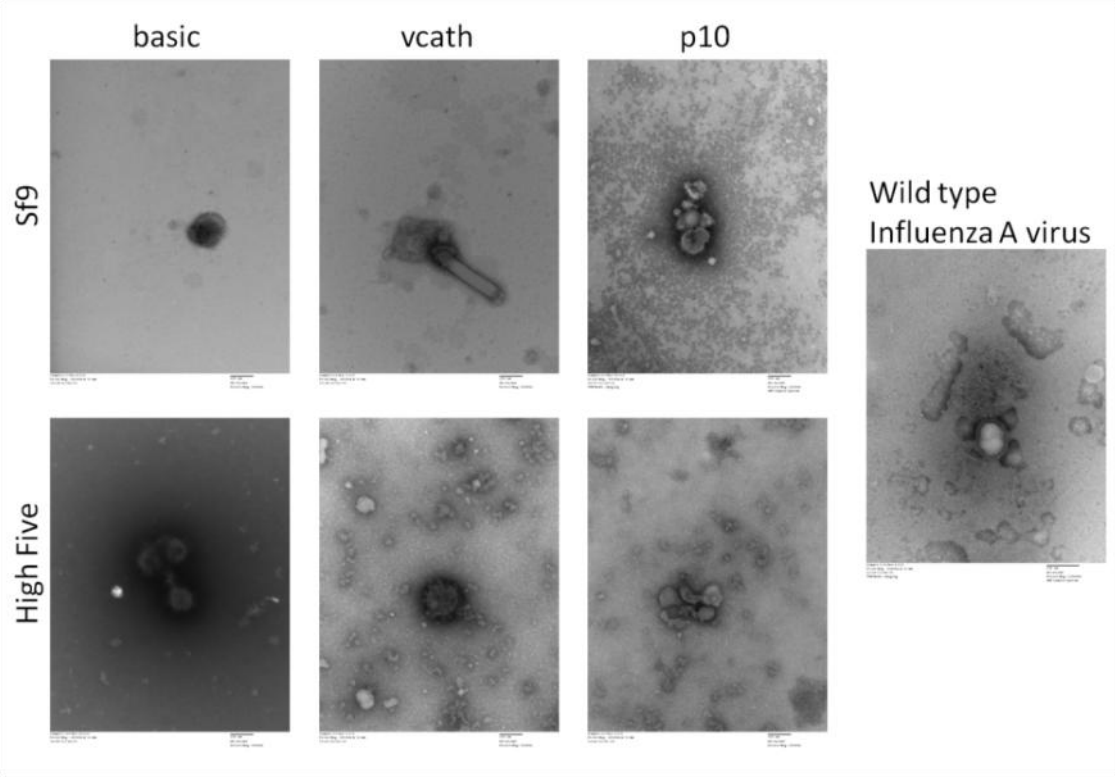
further observation is the presence of very clear localization of proteins at polar opposite ends in some cells (Figure 6-4, white arrows). This type of polarization could be seen in Sf9 cells infected by all three different vectors. Protein localization is less obvious in High Five™ cells, which could be due to the vastly greater amount of protein being produced in these cells. The large amount of protein being produced can be seen using flow cytometry (Figure 6-2). This was also observed by simple visual inspection of the shake flasks. Flasks containing infected High Five™ cells showed far more intense red coloration than flasks with infected Sf9 cells. The relative lack of localization of M1-RFP was also seen in some of the Sf9 cells infected with the vcath construct, and may also be due to the presence of large amounts of M1-RFP protein.



6-4: Confocal microscopy images of Sf9 and High Five™ cells infected with baculoviruses expressing HA-GFP and M1-RFP proteins at 48 hours post infection

6.4.2 Extracellular analysis: the ones that made it out

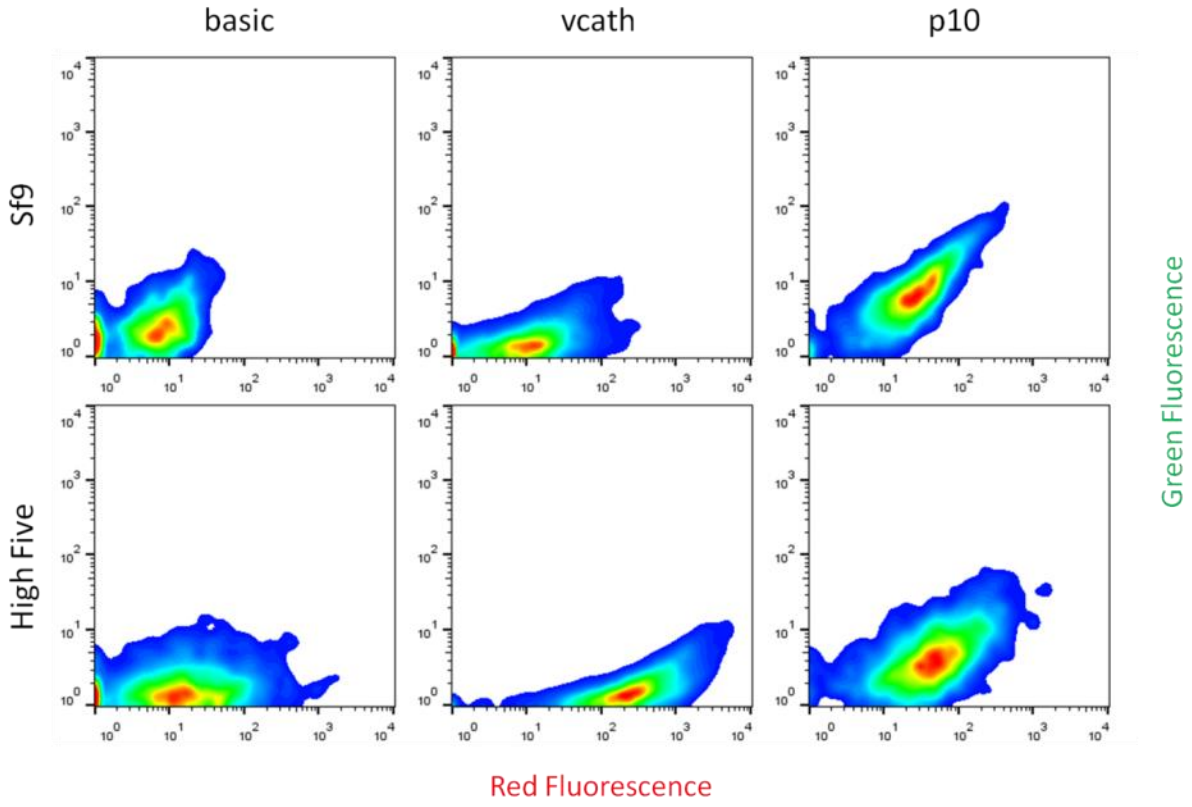
Transmission electron microscopy (TEM) was used to image concentrated supernatant samples from infected insect cells infected by each of the constructs, and showed the presence of VLPs in all cases (Figure 6-5).



6-5: Electron micrograph pictures of influenza VLPs present in supernatant of Sf9 and High Five™ cells infected with the basic, vcath and p10 constructs. The bottom panel shows aggregates present in the concentrated supernatant of p10 infected High Five™ cells

Using flow cytometry, fractions from the iodixanol gradient were examined for particles exhibiting red and green fluorescence (Figure 6-6). The relative red and green fluorescence of the sample differed based on the construct, and generally correlated to the amount of protein produced in the cells when infected with each construct. For instance, iodixanol fractions recovered from cultures infected with the vcath constructs contained particles with low levels of green fluorescence, but high levels of red fluorescence which corresponds to what is seen in the

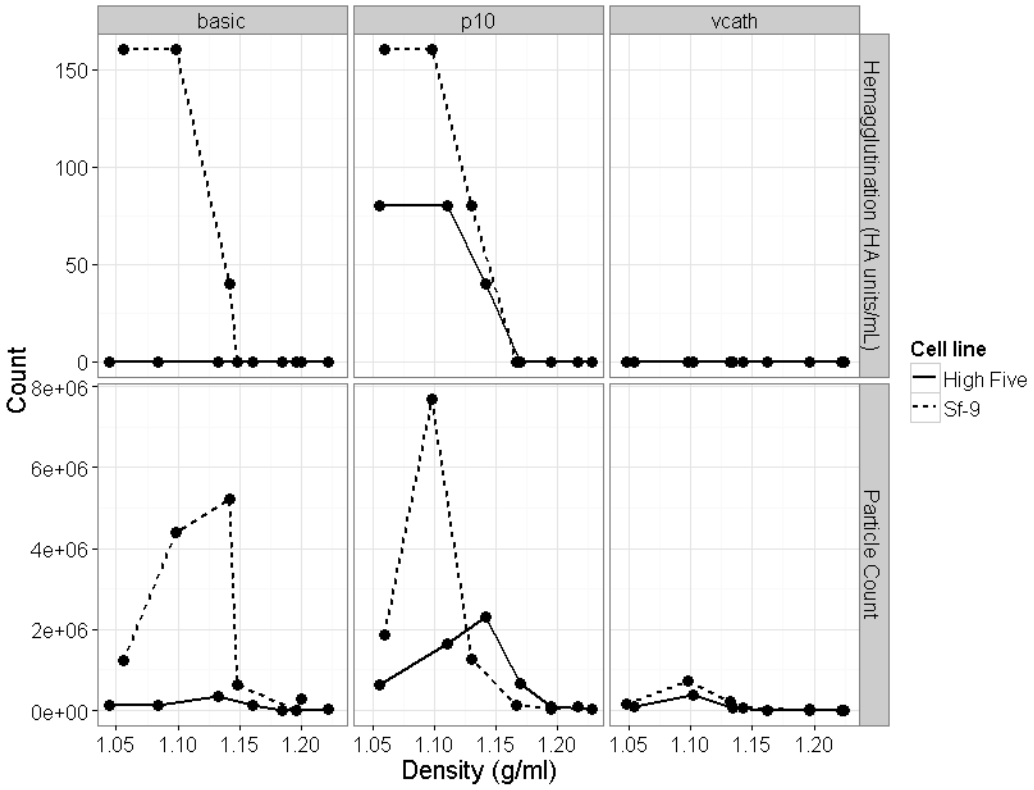
cells in culture. The p10 construct, which produces high levels of HA-GFP, but lower levels of M1-RFP compared to the vcath construct also showed a corresponding change in the relative fluorescence of the particles in purified culture supernatant. The same relationship was seen in the supernatant of the basic construct. VLPs produced from the High Five™ cells had higher red fluorescence than those from Sf9 cells. VLPs produced from cultures infected with the p10 and basic vectors showed higher levels of green fluorescence when produced in Sf9 cells compared to High Five™ cells.



6-6: Flow cytometry scatterplots of ultracentrifuge purified supernatant samples produced from Sf9 and High Five™ cells infected with the basic, p10 and vcath constructs.

The ability to detect particles using flow cytometry also allowed for quantification of particle titers in ultracentrifugation purified sample fractions using a flow cytometry bead standard of known concentrations (Figure 6-7). The hemagglutination assay was also used as a

titration method to determine concentrations of particles showing hemagglutination activity. Comparison of the two methods showed that most of the particles and hemagglutination activity was concentrated in the three lightest ultracentrifugation samples.



6-7: Hemagglutination activity and flow cytometry particle counts of ultracentrifuge-purified fractions of Sf9 and High Five™ insect cell culture supernatant infected with the basic, vcath and p10 constructs

The highest levels of hemagglutinin activity were present in the supernatants of p10 and basic infected Sf9 cells, with the p10 infected High Five™ cells showing relatively lower activity. However, all three supernatants showed similar titers of fluorescent particles when examined by flow cytometry. The basic construct does not show hemagglutination activity in High Five™ supernatant. Only very low numbers of fluorescent particles were produced by the vcath construct in Sf9 cells. Low VLP titers were also seen with High Five™ cells infected with basic and vcath constructs. Based on our flow cytometry results, we estimate the volumetric

productivity of the p10 baculovirus infected Sf9 cultures to be nearly 8×10^5 VLP particles per mL of culture volume, while the High Five™ cultures only produced only about 2.3×10^5 particles/mL when infected with the same baculovirus. The basic baculovirus infected Sf9 cells produced particles at a titer of nearly 4.3×10^5 VLPs per mL of culture volume.

6.5 Discussion

The control of production of multiple proteins within the baculovirus-insect cell system has been shown to be a non-trivial process, where the levels of expression of one gene can affect the expression of other genes in a simple two fluorescent protein production system (George et al, 2015). This work examines this effect in a more complex and industrially relevant system producing two fluorescently tagged influenza proteins in the insect cell system which then assembles into VLPs.

Examination of influenza protein production in cells by flow cytometry revealed that this effect can indeed be seen in this work as well, where the high HA-GFP producing p10 construct expresses M1-RFP to a much lower level than the low HA-GFP producing vcath construct. The basic construct, which is supposed to drive high level expression at the late phase of infection, only seems to drive a moderate level of HA-GFP expression, but also has a low level of M1-RFP expression. In a previous work (George et al., 2015), which used almost identical constructs to drive expression of unfused *GFP* and *RFP* genes, the *basic* promoter did drive high level expression of the *GFP* gene, and produced low levels of RFP. It is a possibility that in our current constructs, the early expressed HA-GFP protein product is being rapidly recruited to the

membrane and then sent out of the cell, thereby reducing cell associated green fluorescence in the basic construct.

6.5.1 HA-GFP and M1-RFP production in Sf9 and High Five™ cells

The data from Figure 6-2 was used to generate expression ratios of the two fluorescent proteins produced from the different constructs in Sf9 and High Five™ cells over time. This Figure 6-3 is analogous to the figure in a previous work (George et al, 2015) showing the ratios of unfused GFP and RFP produced in Sf9 cells over time. This shows that the protein expression ratios obtained in the simple GFP and RFP system are also applicable in the more complicated HA-GFP and M1-RFP coexpression system. This in turn gives evidence that the expression ratios seen in these two systems are applicable generally while considering protein coexpression in BEVS

Examination of green and red fluorescence over time in Figures 6-2 showed that in general all baculovirus constructs produce higher levels of fluorescent proteins in High Five™ cells as compared to Sf9 cells. The one notable exception to this rule was the basic construct, where HA-GFP was produced at the same levels in both High Five™ and Sf9 cells. This can be clearly seen in Figure 6-3, where the expression ratios of HA-GFP and M1-RFP are the same for Sf9 and High Five™ cells, except in the case of expression driven by the basic construct.

The analysis of influenza protein production in insect cells using this method is made more difficult by the fact that the proteins being produced are continually being trafficked to the viral membrane and exported out of the cell. The rate of export of proteins may vary based on the phase of baculovirus infection; therefore, interpretation of the flow cytometry data has to take this into account. For instance, the level of HA-GFP production from the basic construct in High

Five™ cells could be influenced by the export of the protein from the cell. Alternatively, the level of expression seen from the basic promoter could be accurate and representative, and could be one reason for the well-known poor amplification of AcMNPV virus in High Five™ cells (Krammer et al, 2010). However, as seen in Figure 6-2, levels of protein expression driven by the late vcath and very late p10 promoters are much higher in High Five™ cells, as compared to Sf9 cells. Taken together, these results indicate that late and very late phase protein production driven by baculovirus promoters such as *vcath* and *p10* are very active in High Five™ cells when compared to Sf9 cells, but the same may not be true of earlier promoters such as the baculovirus *basic* promoter.

Confocal microscopy of infected insect cells showed that HA-GFP is generally localized to the cell membrane in all constructs, while M1-RFP localizes to either the cell membrane, or around the nucleus. However, in the vcath construct, the M1-RFP protein is spread out through the cell, which could be because of the much larger amount of M1-RFP protein produced by this construct. Protein localization is not as obvious in High Five™ cells, and could because of the large amount of influenza protein produced in these cells (Krammer et al., 2010). Baculovirus infection of insect cells can arrest the cell cycle in either the S or G2/M phase (Braunagel et al., 1998; Ikeda and Kobayashi, 1999; Saito et al., 2002) and the localization of HA-GFP protein to polar ends of some Sf9 cells could be indicative of polarization of the cell membrane during one of these phases.

6.5.2 Influenza VLPs are Exported into Culture Supernatant

The works listed previously (Chapter 5) shows that influenza VLPs can bud out of insect cells and be exported to the supernatant. In our experiments, we detected influenza VLPs from all constructs by transmission electron microscopy (TEM). The formation of VLPs from Sf9 and

High Five™ cells infected with the vcath vector is of note given that HA-GFP is expressed at very low levels and M1-RFP at very high levels. Therefore, it is not clear if the VLPs seen from infection with the vcath construct are the result of M1-RFP associating with HA-GFP, or by M1-RFP budding out alone from the insect cells (Gómez-Puertas et al., 2000).

6.5.3 Influenza VLP Composition and Titer is Influenced by Levels of Component Proteins in Cells

A second aspect of this work was to attempt to control influenza VLP composition by varying levels of HA-GFP and M1-RFP produced in cells. Flow cytometry detection of influenza particles from the various constructs showed that the expression levels of protein within the cells is translated into differing VLP compositions of HA-GFP and M1-RFP. The vcath construct produced particles with very high red fluorescence, but low green fluorescence, and this, coupled with the lack of any detectable hemagglutination activity means that it is plausible that these particles only consist of M1 protein, which has been shown to be capable of mediating budding in the baculovirus insect cell system (Gómez-Puertas et al., 2000). However, the numbers of particles produced as a result of infection with the vcath construct, and detected by flow cytometry, was far less than in the case of the basic and p10 construct, which may be because of reduced budding efficiency in the absence or scarcity of HA protein.

The basic and the p10 constructs infecting Sf9 cells produced similar numbers of VLP particles as seen by flow cytometry; and, hemagglutination assays conducted on ultracentrifugation fractions of baculovirus supernatant revealed that the purified basic and p10 supernatants had almost identical hemagglutination activity. This, despite that p10 construct infected cells accumulated more protein intracellularly, and had a higher level of VLP fluorescence (per VLP) as detected by flow cytometry. Taken together these facts indicate that

the lower level of HA-GFP expression in the basic construct does not seem to reduce particle formation to a great extent, and the early expression of the HA-GFP under the control of the basic promoter may speculatively allow for efficient HA-GFP protein export and VLP production at an earlier stage, even with lowered HA-GFP expression. It would be worthwhile to see if VLP titers could be improved by increasing the levels of expression of HA-GFP during the early stage of the infection process.

6.5.4 Sf9 Cells are More Efficient VLP Producers than High Five Cells

The p10 construct produced comparable levels of fluorescent particles and hemagglutination in High Five™ and Sf9 supernatant, even though there was far more HA-GFP and M1-RFP produced in High Five™ cells. This result is supported by evidence from other works (Krammer et al., 2010) where it was found that High Five cells produce the same number of VLP particles as Sf9 cells in spite of producing far more protein intracellularly. Another result is that the basic construct infecting High Five™ cells resulted in very few particles in the supernatant, even though both the Sf9 and High Five™ cells produce similar quantities of both HA-GFP and M1-RFP, which indicates that High Five™ cells are not as good as Sf9 cells for the production of VLPs.

Much of the recombinant protein produced in the cells, especially at the very late stage of the infection process, is not efficiently incorporated into VLPs, and the titers of VLPs are extremely low, only on the order of 10^6 particles/ml. This is especially apparent in the High Five™ cells, which in general produce far more protein from the late and very late p10 and vcath promoters, but do not produce more VLPs than Sf9 cells. The efficient export of HA-GFP from Sf9 cells infected with the basic construct provides some insight into improving this system, as even low levels of HA-GFP expression by the basic construct produce similar levels of VLPs as

when HA-GFP is produced at much higher levels at the very late phase by the p10 construct. Expressing all VLP component proteins at high levels during the early or late stage of the infection process may improve VLP production dramatically. This would also mimic the baculovirus budding process, where most of the major structural components of the budded baculovirus are produced at the late stage of infection, followed by budding in the late and very-late phases (Matindoost et al., 2015).

6.5 Conclusions

While the manipulation of protein expression ratios to influence particle compositions has been explored in previous studies (Hu and Bentley, 2001), this study is the first to systematically vary expression levels of influenza HA and M1 proteins to examine their incorporation into VLPs. In addition, the use of tagged component proteins allowed the rapid detection of protein ratios in VLPs through the use of a direct technique like flow cytometry which examines the fluorescence of each individual particle, as opposed to an indirect technique such as measuring the ratios of component proteins in purified virus preparations by Western blotting. It was found that the levels of modified influenza HA-GFP and M1-RFP proteins could be controlled in insect cells through the use of different promoter combinations, and that this affected the composition of the final VLP product.

The rational design of expression profiles of different VLP component proteins in a cell, with controlling gene expression levels, would allow for sophisticated control of VLP production within the cell. It is hoped that the concepts explored in this work will be extended to the study of other VLP systems with the end goal of improving VLP production in cell culture systems.

Chapter 7 : Effect of expression of copper-zinc superoxide dismutase in baculovirus-infected insect cells

7.1 Introduction

The infection of insect cells by baculovirus causes oxidative stress within the cell (Wang et al., 2001b), which may be due to the production of reactive oxygen species such as peroxides (O_2^{2-}) and superoxides (O_2^-) from a variety of mechanisms. These cause significant damage to the cell through DNA damage, lipid peroxidation, and protein oxidation (Farinati et al., 1999; Wang et al., 2001a). These reactive oxygen species can be deactivated by several antioxidant enzymes within the cell. In insect cell lines, these include superoxide dismutases, which convert highly reactive superoxide species into peroxides, and enzymes such as catalase and ascorbate peroxidase (Wang et al., 2001a).

There are two types of superoxide dismutase (SOD) enzymes in Sf9 and High Five™ insect cells: copper-zinc SOD (Cu,Zn-SOD) in the cytoplasm and manganese SOD (Mn-SOD) in the mitochondria. Mn-SOD contributes to a larger portion of the SOD activity in High Five™ compared to in Sf9 cells (Wang et al., 2001a). The activity of Mn-SOD is not significantly affected by baculovirus infection (Wang et al., 2001b); however, Cu,Zn-SOD activity decreases in both Sf9 and High Five™ cells following infection (Wang et al., 2001b). Low level expression of human Mn-SOD expression results in a 20% increase in viability at 72 and 96 hours post infection in insect High Five™ cells, but provides no protection in Sf9 cells (Wang et al., 2004).

Although an increase in viability has been attributed to the production of human Mn-SOD in insect cells, it is not clear whether or not the control truly allowed for an appropriate comparison or isolation of the manipulated variable. In the study by Wang et al. (2004), control

cells were infected with a baculovirus expressing very high levels of a control protein, while the cells with increased viability were subjected to a baculovirus vector carrying the *SOD* gene under a weak early promoter.

The lack of protective effect in Sf9 cells is not easily understood, but has been speculated to be due to the large amount of peroxide that is generated by SOD, which cannot be disposed of by the relatively small amount of catalase and ascorbate peroxide in Sf9 cells (Wang et al., 2001a). The difference in effects on Sf9 and High Five^{TM} cells could also be because most of the superoxide defense in Sf9 cells comes from Cu,Zn-SOD and that it is down regulated upon baculovirus infection.

Given that expression of superoxide dismutase could enhance overall recombinant protein production, we decided to follow-up on the study of Wang et al. (2004) but with an eye to using our alternative promoter polycistronic baculovirus system. In this work, we produced two constructs that co-express Cu,Zn-SOD under the control of either a weak *gp64* or strong *p10* promoter, and DsRed2 red fluorescent protein (RFP) under the control of a very strong polh promoter.

7.2 Materials and Methods

7.2.1 Baculovirus Vector Construction

Two vectors both expressing red fluorescent DsRed2, and copper-zinc superoxide dismutase (Cu,Zn-SOD) were constructed based on plasmid constructs described in from Chapter 3. Briefly, a modified human Cu,Zn-SOD sequence was isolated from plasmids provided by Dr. Elizabeth Meiering of the Department of Chemistry, at the University of Waterloo, using the

forward and reverse primers 5’ – CAGCTGAGCGCCGGTCGCTACCATTACCAGATGGCCACAAAGGCTGTTGCT – 3’ and 5’-CCGAGTTTGTCAGAAAGCAGACCAAACAGCGGTTATTGGGCGATCCCAATTACACC AC – 3’ . Underlined portions of the primer sequences correspond to regions on the pAcUW51 plasmid downstream of the p10 promoter. This DNA fragment was then cloned into baculovirus transfer vector p10-GFP-RFP and gp64-GFP-RFP (George et al., 2015), which in turn was based on plasmid pAcUW51 (BD Pharmingen, San Diego, CA) using overlap extension PCR (Bryksin and Matsumura, 2010). The resulting plasmid contained the SOD gene under control of either a baculovirus *p10* or *gp64* promoter and the RFP gene under the control of a baculovirus *polh* promoter. Baculoviruses were generated from these vectors using the BD BaculoGold™ Transfection Kit (BD Pharmingen). The original p10-GFP-RFP and gp64-GFP-RFP baculoviruses expressing GFP either under a *p10* or *gp64* promoter, and RFP from a very late *polh* promoter were used as controls in this experiment (George et al., 2015).

7.2.2 Cells and virus amplification

Spodoptera frugiperda clonal isolate 9 (Sf9) cells (GIBCO, Carlsbad, CA, USA) were maintained in capped glass Erlenmeyer flasks in SF900III media (GIBCO, Carlsbad, CA, USA) at a temperature of 27 °C on an orbital shaker rotating at 130 rpm. Maintenance cell cultures were kept at a density between 0.5 and 4 $\times 10^6$ cells/ml.

Baculovirus amplification was conducted by seeding cultures at 0.5×10^6 cells/ml, allowing them to grow to ~$3x10^6$cells/ml to reach a mid-exponential phase, diluting to 1×10^6 cells/ml in fresh media and infecting with various viruses. The cultures were allowed to proceed until the viability dropped to 70–80%, after which the culture medium was harvested and

centrifuged at 1000 × g for 10 min in order to spin down cells and cell debris. The supernatant was removed and used as baculovirus stock for experiments

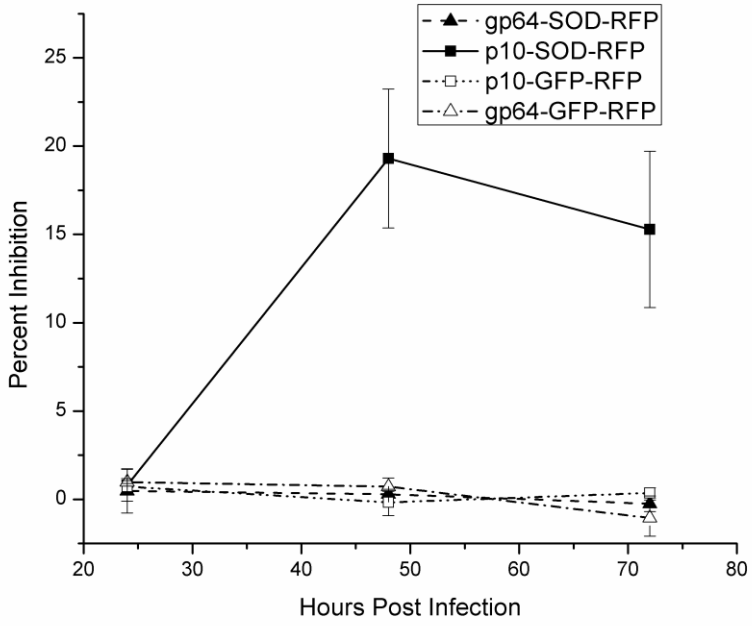
7.2.3 SOD activity measurement

Cu,Zn-SOD activity was measured in the cell lysates using a pyrogallol assay in which Cu,Zn-SOD inhibits the autoxidation of pyrogallol (1,2,3-Benzenetriol) by superoxide radicals in solution, which can be measured as an inhibition in the rate of increase of absorbance at 420 or 325 nm (Marklund and Marklund, 1974). Bovine liver catalase is added to the reaction mixture and has no effect on the autoxidation of pyrogallol, but increases the reaction efficiency by halving the amount of oxygen consumed in the reaction through the evolution of molecular oxygen from hydrogen peroxide generated by Cu,Zn-SOD (Marklund and Marklund, 1974).

Cell lysates from samples taken 48, 72, or 96 hpi were added to a 96-well plate on ice. Unless otherwise noted, 2.5 µg of cell lysate was added to each reaction. Assay buffer (50 mM Tris, 1 mM diethylenetriaminepentaacetic acid, pH 8.2) was heated to 25°C on a hot plate and stirred at maximum speed with a magnetic stir bar to promote aeration of the solution for 1 h prior to the experiment. A fresh solution of pyrogallol (8 mM pyrogallol in 10 mM HCl) was prepared in MilliQ water, kept at 4°C, and protected from light. A mixture of 20 mL assay buffer, 200 µL of bovine liver catalase (Sigma-Aldrich, Oakville, ON, Canada) diluted to 100 units/mL in KH_2PO_4 buffer and stored at -80°C until use, and 500 µL of fresh pyrogallol solution (Sigma-Aldrich, Oakville, ON, Canada) was rapidly mixed and 200 µL of the mixture was immediately added to each well of the plate containing cell lysate. Eight absorbance readings were immediately measured at 31 second intervals over 3.5 minutes using a Synergy 2 (Bio-Tek, Winooski, VT, USA) spectrophotometer at 420 or 325 nm. Data was collected using a Synergy4 multi-mode reader (Bio-Tek, Winooski, VT, USA). Linear regression was performed in Excel to

determine the rate of autoxidation of pyrogallol in the presence of the various cell lysates and compared to the reference, which contained no cell lysate. Rates were expressed per unit of total cellular protein

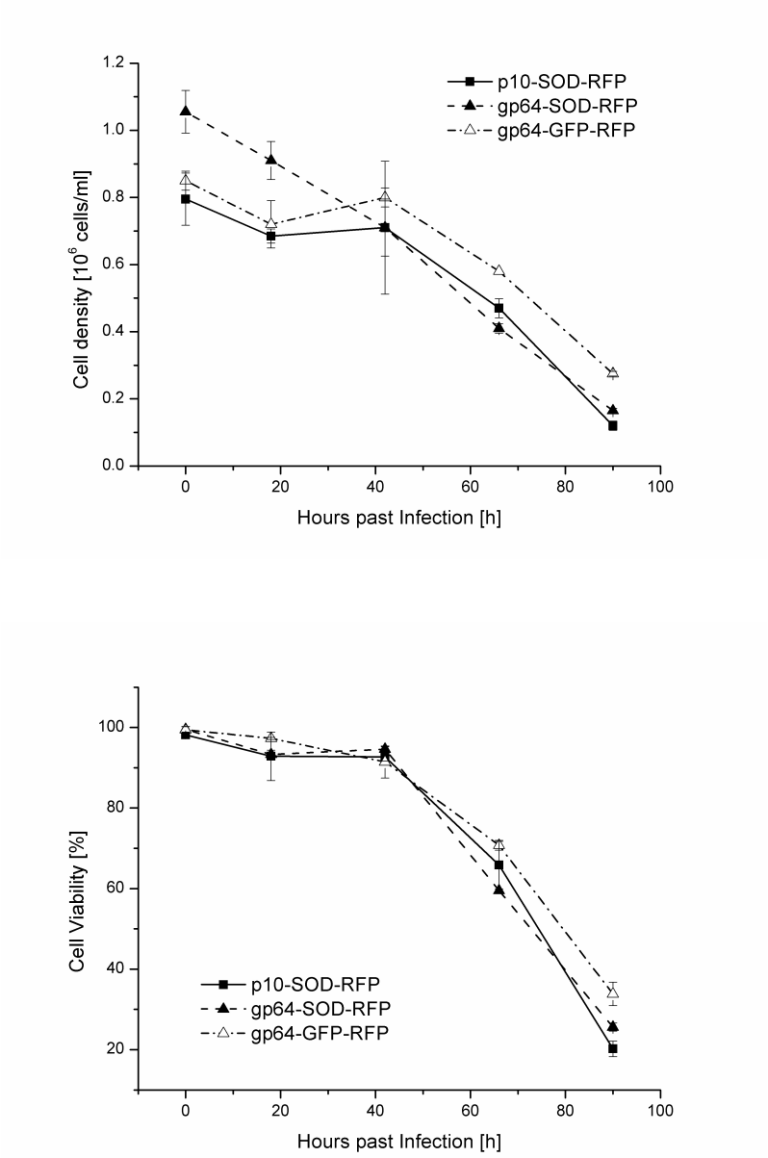
7.3 Results and Discussion



7-1: SOD activity of Sf9 cells infected with various baculoviruses in SF900 III media supplemented with 1.0 mM CuSO4 and 0.1 mM ZnSO4.The Y axis represents percent inhibition of pyrogallol autooxidation per µg of cell lysate. Error bars represent one standard deviation from the mean of duplicate values

A pyrogallol assay (Marklund and Marklund, 1974) was used for the detection of SOD activity in cells infected with the various types of baculovirus. As can be seen in Figure 7-1, cells infected with the non SOD producing virus showed no SOD activity, while cells infected with baculovirus producing SOD under the weak gp64 promoter only showed SOD activity when much larger cell lysate protein amounts were loaded into the SOD activity assay (data not shown). As expected, producing SOD under the control of the very strong p10 promoter resulted in easily detectable SOD activity within infected cells. These results show that the native levels

of SOD activity within Sf9 cells is quite low, and also confirms that the expressed Cu-Zn SOD is active within infected cells.



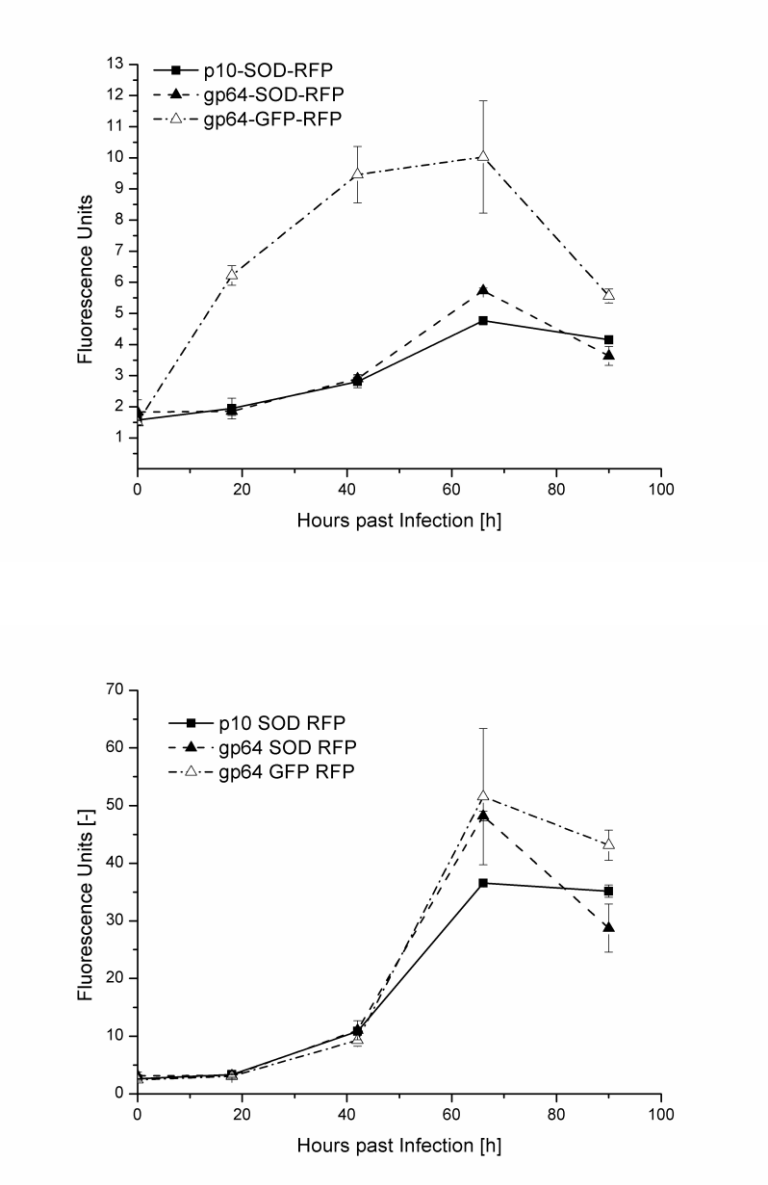
7-2:Cell density (A) and viability (B) of cultures infected with various baculoviruses, in media supplemented with 1.0 mM CuSO4 and 0.1 mM ZnSO4.

A time course of cells infected by the virus at an MOI of 5 indicates that as expected, the cell densities and viabilities decrease over time, with no significant differences between the

different constructs (Figure 7-2). Therefore we conclude that Cu-Zn SOD expression at high or low levels does not prolong the lifespan of baculovirus infected Sf9 cells.

It is possible that reduction of oxidative stress within insect cells through SOD expression would increase the productivity of the cell in terms of fluorescent protein production. Therefore, infected cells were subjected to flow cytometry, and their red and green fluorescence measured (Figure 7-3). As expected, significant green fluorescence was only observed in cells infected with GFP producing virus. The slight increase in green fluorescence from SOD expressing cells over time may be due to either signal leakage from the red fluorescent protein, or from some process that produces fluorescent entities

The levels of RFP produced were similar in the case of constructs where either the GFP or SOD was under the control of the *gp64* promoter. This indicates that there was no enhancement of fluorescent RFP when protective SOD species were present in cells. In addition, the "competition" effect was also seen in this system, as the level of production of DsRed2 was much less when the SOD gene was placed under the control of the very strong *p10* promoter, which confirms results obtained earlier, with the dual fluorescent protein system shown in Chapter 5 (George et al, 2015).



7-3: Green (A) and red (B) fluorescence from the various constructs over time within Sf9 cells infected with various baculoviruses.

The experiments conducted show that the expression of Cu-Zn SOD in Sf9 insect cells had no effect on improving cell viability, or cell productivity. In the future, the effect of Cu-ZnSOD expression in High Five^{TM} cells should also be investigated. In addition, Mn-SOD constructs expressing both Mn-SOD and RFP should be produced, and the effect of their expression should be investigated in both Sf9 and High Five^{TM} cells. The study of Mn-SOD in

High Five™ cells will complement the work by Wang et al (2004) and will determine if Mn-SOD expression improves cell viability when high levels of protein are being produced.

Conclusions and Recommendations

The work presented in this thesis aimed to expand the utility of the baculovirus vector expression system for the simultaneous production of multiple proteins. This work was started with the goal of demonstrating tight control over the timing and expression levels of multiple genes within insect cell, and to examine any effects like "competition" between genes for cellular resources.

This was realized in the first study where two simple fluorescent proteins eGFP and DsRed2 were expressed under the control of various promoter combinations. The expression levels of these two genes could be finely controlled using these promoter combinations, such that we determined the expression ratios of these proteins over time, with different promoter combinations. The study showed that coexpressed genes do in fact compete with each other for resources, and so the expected expression level from a promoter depends on the expression levels of other heterologous and baculovirus genes being expressed. This lays the basis for a theoretical template which other researchers can use to tailor expression systems that can produce proteins at different levels at different times during the baculovirus infection cycle. The genome of the many baculoviruses found in nature provides a rich source of promoters, as well as modified versions of these, that are active at various times and at different levels during the baculovirus infection cycle in insect cells. This study examined only the use of baculovirus promoters, but future research could examine the effects of other genetic elements to improve control over this system. In addition, this study only examined expression from two gene systems and future studies could expand this concept to larger systems with three or more genes. This could especially be relevant for systems which require widely disparate levels of gene expression, such as when low levels of foreign chaperones or proteases are needed to efficiently produce or mature a protein of interest.

This second study was meant to address a question raised by the first study: namely, is the "competition" effect that is seen present at the transcription or translation stages. Real Time qPCR tracking of eGFP and DsRed2 transcripts revealed that the competition effect seemed to be present at the transcription stage, even though this may not be the only source of the competition effect. However, the data obtained was not of high quality.

The third and fourth studies were conducted to extend the work done with eGFP and DsRed2 to a more complex and commercially relevant system: the production of influenza virus-like particles. This system involved the production of influenza hemagglutinin and matrix 1 proteins, that require significant post-translational modification, export to the cell membrane and assembly into VLPs, all features which were lacking from the eGFP – DsRed2 system. The influenza proteins were modified by genetically fusing them to fluorescent proteins to enable easy and direct visualization of influenza protein production in insect cells. This also allowed for examination of fluorescent VLP production into culture supernatant. These modified virus genes were then placed under the control of different promoter combinations and it was again shown that the levels of proteins could be reliably controlled by promoter control, and that competition effects could be observed in this more complex system. In addition, manipulating intracellular influenza protein levels affected the ratio of these proteins in VLPs in culture supernatant. These effects were shown to be present in Sf9 and High Five^{TM} cell lines. Unexpectedly, while High Five^{TM} cells produced far more intracellular protein than Sf9 cells, VLP production was not higher than from Sf9 cells. This work leads to several avenues of improvement. One of the major limitations of this VLP production system is the very low numbers of VLPs formed and exported into culture supernatant. This could be due to several factors such as lack of assembly of influenza proteins into VLPs in insect cells, or lack of VLP release from the cell surface. This

could be a subject of future research. It is also apparent that much of the influenza protein produced in both Sf9 and High Five™ cells are not exported as VLPs and future studies on the bottlenecks in the VLP production process have the potential to greatly improve influenza VLP production from insect cells. The fluorescent influenza proteins produced in this work could also be used to examine influenza protein production in other cell types, and the approach of adding fluorescent tags to virus proteins could be adapted to other VLP forming virus systems.

The last study presented in this work involved expressing Cu,Zn-superoxide dismutase to extend the lifespan of infected insect cells, by reducing oxidative stress through the destruction of superoxide species. It was thought that extending the lifespan of the cells could increase the production of DsRed2, which was coexpressed with SOD. No protective effect of SOD was found in Sf9 cells, and no increases in DsRed2 production were observed. Future research could examine the effect of coexpressing catalase with SOD, which would break down the peroxide species produced by SOD and further reduce oxidative stress in infected insect cells. In addition, while Cu,Zn-SOD was not protective in Sf9 cells, it's expression may be protective in other insect cells such as High Five™ cells.

Taken together, it is hoped that this work demonstrates the potential for the implementation of "designed" coexpression systems in BEVS, where the timing and levels of individual proteins can be pre-determined based on works similar to that presented in Chapter 3. These proteins can be components of a complex protein product, as demonstrated with the production of influenza VLPs, or chaperones, or other helper proteins to improve the production process, as in the case of the coexpression of SOD to improve cell viability.

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Appendix

Gene sequences used in this project

Influenza A virus (A/Puerto Rico/8/1934(H1N1)) HA gene for hemagglutinin,

ATGAAGGCAAACCTACTGGTCCTGTTATGTGCACTTGCAGCTGCAGATGCAGACACA
ATATGTATAGGCTACCATGCGAACAATTCAACCGACACTGTTGACACAGTACTCGAG
AAGAATGTGACAGTGACACACTCTGTTAACCTGCTCGAAGACAGCCACAACGGAAA
ACTATGTAGATTAAAAGGAATAGCCCCACTACAATTGGGGAAATGTAACATCGCCG
GATGGCTCTTGGGAAACCCAGAATGCGACCCACTGCTTCCAGTGAGATCATGGTCCT
ACATTGTAGAAACACCAAACTCTGAGAATGGAATATGTTATCCAGGAGATTTCATCG
ACTATGAGGAGCTGAGGGAGCAATTGAGCTCAGTGTCATCATTCGAAAGATTCGAA
ATATTTCCCAAAGAAAGCTCATGGCCCAACCACAACACAAACGGAGTAACGGCAGC
ATGCTCCCATGAGGGGAAAAGCAGTTTTTACAGAAATTTGCTATGGCTGACGGAGA
AGGAGGGCTCATACCCAAAGCTGAAAAATTCTTATGTGAACAAAAAAGGGAAAGAA
GTCCTTGTACTGTGGGGTATTCATCACCCGCCTAACAGTAAGGAACAACAGAATCTC
TATCAGAATGAAAATGCTTATGTCTCTGTAGTGACTTCAAATTATAACAGGAGATTT
ACCCCGGAAATAGCAGAAAGACCCAAAGTAAGAGATCAAGCTGGGAGGATGAACT
ATTACTGGACCTTGCTAAAACCCGGAGACACAATAATATTTGAGGCAAATGGAAAT
CTAATAGCACCAATGTATGCTTTCGCACTGAGTAGAGGCTTTGGGTCCGGCATCATC
ACCTCAAACGCATCAATGCATGAGTGTAACACGAAGTGTCAAACACCCCTGGGAGC
TATAAACAGCAGTCTCCCTTACCAGAATATACACCCAGTCACAATAGGAGAGTGCC
CAAAATACGTCAGGAGTGCCAAATTGAGGATGGTTACAGGACTAAGGAACATTCCG
TCCATTCAATCCAGAGGTCTATTTGGAGCCATTGCCGGTTTTATTGAAGGGGGATGG
ACTGGAATGATAGATGGATGGTATGGTTATCATCATCAGAATGAACAGGGATCAGG
CTATGCAGCGGATCAAAAAAGCACACAAAATGCCATTAACGGGATTACAAACAAGG
TGAACACTGTTATCGAGAAAATGAACATTCAATTCACAGCTGTGGGTAAAGAATTCA
ACAAATTAGAAAAAAGGATGGAAAATTTAAATAAAAAAGTTGATGATGGATTTCTG
GACATTTGGACATATAATGCAGAATTGTTAGTTCTACTGGAAAATGAAAGGACTCTG
GATTTCCATGACTCAAATGTGAAGAATCTGTATGAGAAAGTAAAAAGCCAATTAAA
GAATAATGCCAAAGAAATCGGAAATGGATGTTTTGAGTTCTACCACAAGTGTGACA
ATGAATGCATGGAAAGTGTAAGAAATGGGACTTATGATTATCCCAAATATTCAGAA
GAGTCAAAGTTGAACAGGGAAAAGGTAGATGGAGTGAAATTGGAATCAATGGGGA
TCTATCAGATTCTGGCGATCTACTCAACTGTCGCCAGTTCACTGGTGCTTTTGGTCTC
CCTGGGGGCAATCAGTTTCTGGATGTGTTCTAATGGATCTTTGCAGTGCAGAATATG
CATCTGA

Influenza A virus (A/Puerto Rico/8/1934(H1N1)) segment 7, coding for the M1 gene

ATGAGTCTTCTAACCGAGGTCGAAACGTACGTACTCTCTATCATCCCGTCAGGCCCC
CTCAAAGCCGAGATCGCACAGAGACTTGAAGATGTCTTTGCAGGGAAGAACACCGA
TCTTGAGGTTCTCATGGAATGGCTAAAGACAAGACCAATCCTGTCACCTCTGACTAA
GGGGATTTTAGGATTTGTGTTCACGCTCACCGTGCCCAGTGAGCGAGGACTGCAGCG
TAGACGCTTTGTCCAAAATGCCCTTAATGGGAACGGGGATCCAAATAACATGGACA
AAGCAGTTAAACTGTATAGGAAGCTCAAGAGGGAGATAACATTCCATGGGGCCAAA
GAAATCTCACTCAGTTATTCTGCTGGTGCACTTGCCAGTTGTATGGGCCTCATATACA
ACAGGATGGGGGCTGTGACCACTGAAGTGGCATTTGGCCTGGTATGTGCAACCTGTG
AACAGATTGCTGACTCCCAGCATCGGTCTCATAGGCAAATGGTGACAACAACCAAT
CCACTAATCAGACATGAGAACAGAATGGTTTTAGCCAGCACTACAGCTAAGGCTAT
GGAGCAAATGGCTGGATCGAGTGAGCAAGCAGCAGAGGCCATGGAGGTTGCTAGTC
AGGCTAGACAAATGGTGCAAGCGATGAGAACCATTGGGACTCATCCTAGCTCCAGT
GCTGGTCTGAAAAATGATCTTCTTGAAAATTTGCAGGCCTATCAGAAACGAATGGGG
GTGCAGATGCAACGGTTCAAG

eGFP from plasmid pcDNA3-GFP

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCT
GGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGAT
GCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTG
CCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTAC
CCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTC
CAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGT
GAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCA
AGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAA
CGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCC
GCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACC
CCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCC
GCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGT
GACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAA

DsRed2 from plasmid pCALNLDsRed

ATGGCCTCCTCCGAGAACGTCATCACCGAGTTCATGCGCTTCAAGGTGCGCATGGAG
GGCACCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTA
CGAGGGCCACAACACCGTGAAGCTGAAGGTGACCAAGGGCGGCCCCCTGCCCTTCG
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HA-GFP-RFP-M synthesized sequence

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GATCCGGTTATTAGT

Color Legend:

Hemagglutinin gene sequence

Matrix 1 gene sequence

eGFP sequence

DsRed2 sequence

polh promoter

p10 promoter

Linker regions

Sequences overlapping baculovirus vector

Baculovirus Quantification Methods

Virus sample preparation for real-time PCR

Viral DNA template was prepared by subjecting samples of baculovirus-containing supernatant to freeze/thaw cycles and Triton X-100 treatments, as detailed previously (George et al., 2012). Briefly, 10% Triton X-100 (EMD Chemicals, Gibbstown, NJ, USA) was added to 200ul cell culture supernatant samples to a final concentration of 0.1% Triton X-100. The samples were incubated at 37 °C for 10 minutes, followed by two freeze/thaw cycles alternating between -80 °C and 37°C, 10 minutes at a time. Treated samples were then used for real-time PCR.

Real-time PCR virus quantification

All real-time PCRs were conducted with a StepOnePlus Real-Time PCR system (Applied Biosystems, Burlington, ON, Canada) and were prepared in MicroAmp Fast Optical 96-well Reaction Plates (Applied Biosystems, Burlington, ON, Canada). Each reaction consisted of 2 µl sample, 10 µl of 2× Power SYBR® Green PCR Master Mix (Applied Biosystems, Burlington, ON, Canada), 900 nM each of forward and reverse primers, and nuclease free water, for a final volume of 20 µl. Replicate samples were prepared in one well and then distributed among the required number of wells. The plate was then sealed with MicroAmp Optical Adhesive Film (Applied Biosystems, Burlington, ON, Canada) and centrifuged briefly at 1000 g in an Eppendorf 5804R centrifuge (Eppendorf Canada, Mississauga, ON, Canada) with an A2-DWP flat plate rotor (Eppendorf Canada, Mississauga, ON, Canada). The primers used for the reactions have been described in another work (Hitchman et al., 2007) and are targeted against a region in the gp64 gene (gp64F – 5- CGGCGTGAGTATGATTCTCAAA – 3’ and gp64R – 5’ – ATGAGCAGACACGCAGCTTTT – 3’).The reaction was conducted according to conditions described previously (George et al., 2012). The initial denaturation was conducted at 95 °C for

10 min, followed by 45 cycles of denaturation at 95 °C for 30 s and annealing/extension at 60 °C for 30 s. Following PCR, a melt curve analysis was performed by heating the final mixture to 95 °C for 15 s followed by annealing at 60 °C for 1 min. The temperature was then increased in 0.3 °C increments to 95 °C for 15 s, with fluorescence being measured during the elevation stage. Each sample was run in triplicate on a single PCR plate to provide statistical validity and confidence in the data obtained. Data obtained from each reaction plate was analysed by StepOne™ Software v2.0. Plasmid standards were generated in our lab, and have been described previously (George et al., 2012). Plasmid standard dilutions were run alongside the samples, and a standard curve of threshold cycle (CT) vs standard concentration was generated. The threshold fluorescence level was automatically set by the instrument control software StepOne™ Software v2.0 (Applied Biosystems, Burlington, ON, Canada) to be significantly higher than the background noise. The CT values of the samples were then compared with the standard curve to determine the concentration of baculovirus DNA in samples.

Baculovirus particle titer determination by flow cytometry

Baculovirus particle counts were conducted using flow cytometry, as described previously (Shen, et al. 2002). Counts were carried out using a BD FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA), and Flow-Set™ Fluorospheres (Beckman Coulter Inc., Fullerton, CA, USA) were used as an external standard to calibrate the instrument and allow for particle concentration determination.

Viable baculovirus titer determination by end point dilution assay

The end point dilution assay was conducted as per the Tissue Culture Infectious Dose 50 (TCID50) assay (Reed and Muench, 1938) and analyzed using equations from King and Possee (King and Possee, 1992). Briefly, a 96 well plate was seeded with 2 x 10^4 cells. Serial dilutions

of baculovirus in Sf900 III media were made ranging from 10^{-4} to $10^{-10}$and added to the plated cells (with each virus dilution being added to 12 wells). The plates were stored at 27^0C for 7 days, after which the plate was examined under a fluorescence microscope, and the wells scored as infected or non-infected based on fluorescence from baculovirus infected wells. The values obtained from the End Point Dilution Assay were used to determine the amounts of virus solution to be added to cell cultures (given the close agreement between methods and stocks).

Viable baculovirus titer determination by growth cessation assay

This assay was used to confirm the results obtained from the End Point Dilution Assay. Briefly, 30 ml cell cultures at 1 x 10^6 cells/ml were infected with 1000, 100, 10, 5 and 1 µl of virus stocks and the cultures were monitored over 5 days, to determine when cell growth cessation occurred. The amount of baculovirus that caused an immediate cessation of growth was taken to be equivalent to an MOI of 3, from which the titre of the virus stock could be determined.

Table A-1: Primers used in work performed in Chapter 3 (Integrated DNA Technologies, Coralville, IA, USA). The underlined regions correspond to sequences on the pAcUW51 plasmid where sequences are to be inserted

Description	Primer Sequence	Primer Name
Primers for amplifying out GFP gene and inserting it into plasmid pAcUW51	5' –CAGCTGAGCGCCGGTCGCTACCATTACCAGATGG TGAGCAAGGGCGAGGAGCTG – 3'	GFPF
	5' – CCGAGTTTGTCAGAAAGCAGACCAAACAGCGGT TACTTGTACAGCTCGTCCATGCCGAGAG – 3'	GFPR
Primers for amplifying out RFP gene and inserting it into plasmid pAcUW51	5' –CTATAAATACGGATCATGGCCTCCTCCGAGAACG TCATC – 3'	RFPF
	5'- GTACTAATAACCGGATCCTACAGGAACAGGTGGTG GCG – 3'	RFPR
Primers for amplifying out the ie1 promoter, and replacing the p10 promoter in pAcUW51	5' - CAACAAGGGGGACTATGAAATTATGCATTTGAGG ATGCGCCATTAGGGCAGTATAAATTG – 3'	ie1F
	5' –CTGGTAATGGTAGCGACCGGCGCTCAGCTGGAATTC AGTCACTTGGTTGTTCACGATC – 3'	ie1R
Primers for amplifying out the basic promoter, and replacing the p10 promoter in pAcUW51	5' – CAACAAGGGGGACTATGAAATTATGCATTTGAGG ATGCCGTTTTGCGACGATGCAG – 3'	basicF
	5' – CTGGTAATGGTAGCGACCGGCGCTCAGCTGGAATT CGTTTAAATTGTGTAATTTATGTAGCTGTAATT – 3'	basicR
Primers for amplifying out the gp64 promoter, and replacing the p10 promoter in pAcUW51	5' – CAACAAGGGGGACTATGAAATTATGCATTTGAGG ATGTGTGTCACGTAGGCCAGATAAC – 3'	gp64F
	5' – GTAATGGTAGCGACCGGCGCTCAGCTGGAATTCC TTGCTTGTGTGTTCCTTATTGAAGCC -3'	gp64R
Primers for amplifying out the vcath promoter, and replacing the p10 promoter in pAcUW51	5' –GGACTATGAAATTATGCATTTGAGGATGAATTTATC TTAATTTTAAGTTGAATTCCAGCT – 3`	vcathF
	5' – GGTAGCGACCGGCGCTCAGCTGGAATTCAACTTA AAATTAAGATAAATTCATCCTC – 3'	vcathR
Primers for amplifying out GFP gene and inserting it into plasmid pFastBac1	5' - CGCGGATCCATGGTGAGCAAGGGCGAGGA - 3'	GFPFB1F
	5' - GCCGAATTCTTACTTGTACAGCTCGTCCATGCCGAG - 3'	GFPFB1R
Primers for amplifying out RFP gene and inserting it into plasmid pFastBac1	5' - CGCGGATCCATGGCCTCCTCCGAGAACGTCA - 3'	RFPFB1F
	5' - GCCGAATTCCTACAGGAACAGGTGGTGGCG - 3'	RFPFB1R

Supplementary

Figures

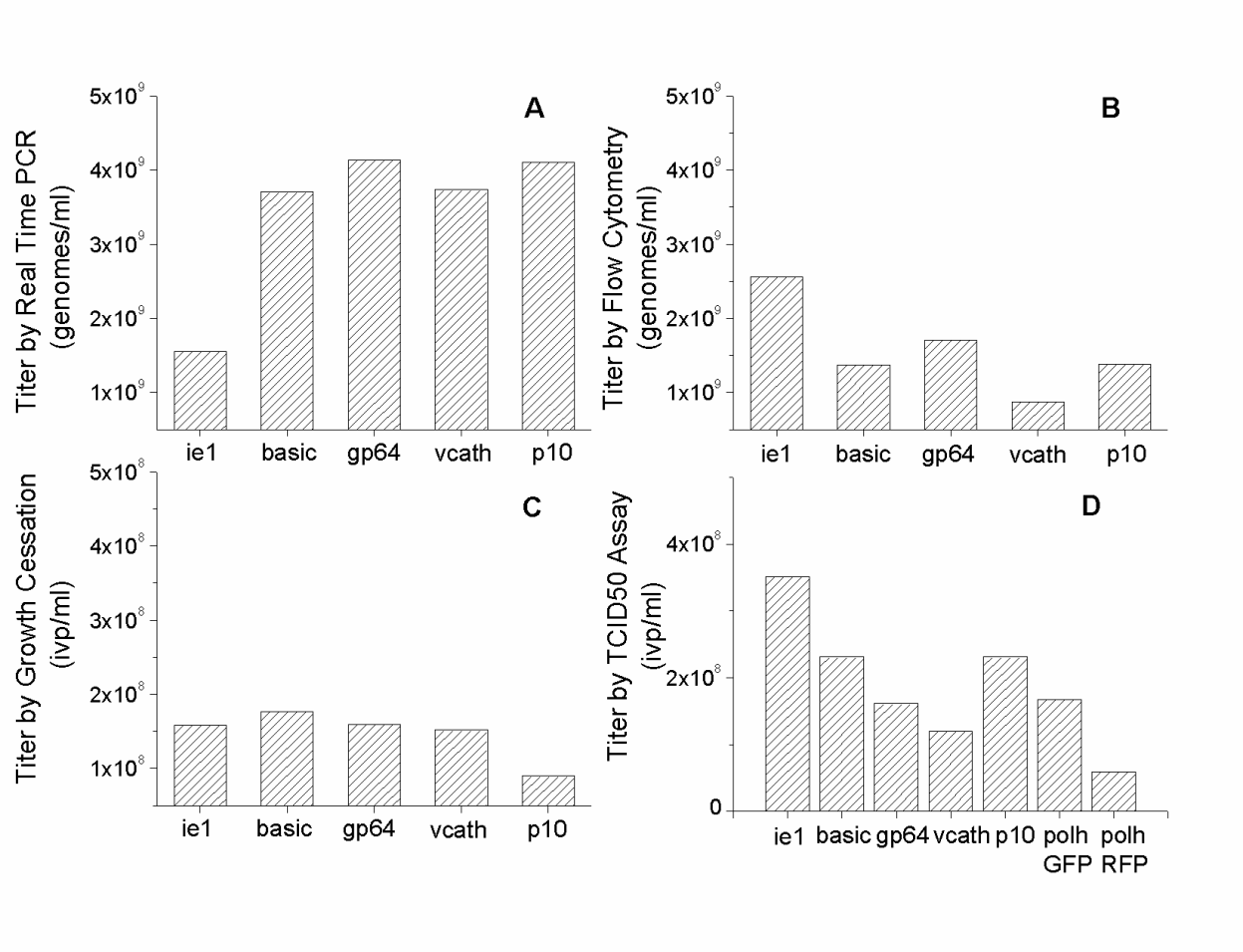

Figure A-1: Comparison of virus titers obtained using four different methods to quantify the five viruses used in this experiment: Real Time PCR (Figure A-1 A), Flow Cytometry (Figure A-1 B), End Point Dilution Assay (Figure A-1 C), and growth cessation assay (Figure A-1 D)

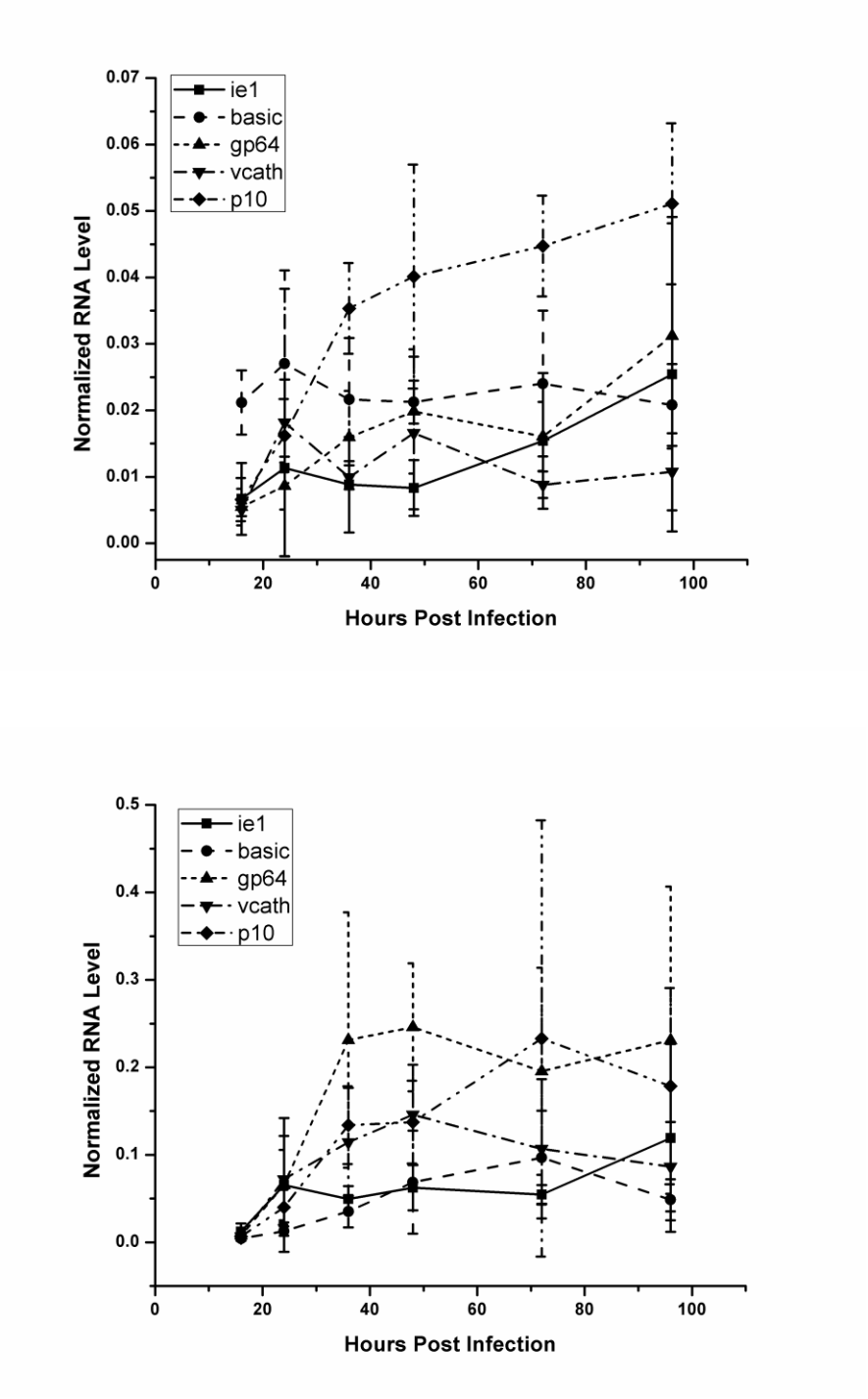

Figure A-2 : eGFP (A-1 A) and DsRed2 (A-1 B) RNA levels in cells infected with different baculovirus constructs, normalized with respect to cellular 28SrRNA levels