Comparing influenza virus hemagglutinin (HA) expression in three different baculovirus expression systems

by

Alexandra Elliott

A thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Master of Science
in
Molecular and Cellular Biology

Guelph, ON, Canada
©Alexandra Elliott, August, 2012
ABSTRACT

COMPARING INFLUENZA VIRUS HEMAGGLUTININ (HA) EXPRESSION IN THREE DIFFERENT BACULOVIRUS EXPRESSION SYSTEMS

Alexandra Elliott
University of Guelph, 2012

Advisor: Professor P.J. Krell
Co-advisor: Dr. Éva Nagy

In this study, the expression of HA, a key immunogenic protein of influenza viruses, in insect cells was compared using three baculovirus expression strategies: protein over-expression, surface (GP64) display, and capsid (VP39) display. Further, a recombinant virus expressing NA, another immunogenic influenza virus protein, was generated and fused to an HA epitope-tag. Western immunoblot using various antibodies, including those against HA, demonstrated the expression of HA and NA for all recombinant viruses. HA showed stronger expression when fused to the C-terminus of VP39 than the N-terminus, but unlike other expression methods, there was no observable cleavage of HA in VP39-displayed viruses. Cells infected with only over-expressed and surfaced-displayed HA were biologically active, and capable of hemadsorption and hemagglutination of chicken red blood cells. These results suggest that GP64 display or over-expression are the most efficacious modes of HA-expression for use as antigen to detect anti-HA antibodies in poultry.
ACKNOWLEDGEMENTS

My gratitude goes to my co-advisors, Dr. Peter Krell and Dr. Éva Nagy for giving me the opportunity to complete my graduate research in their laboratories. I would also like to thank you for your consistent support, advice, and for thoroughly enriching my graduate experience. I would also like to acknowledge Dr. Sarah Wootton, the third member of my committee, for contributing ideas, offering guidance and supporting me throughout this research.

A number of materials were received from colleagues, enabling this research to take place. Thank you to Dr. Gary Blissard at Cornell University in Ithaca NY, for supplying us with the monoclonal anti-GP64 antibody. Thank you to Dr. Congyi Zheng and Qingzhen Liu (Wuhan University, Wuhan, China) for supplying us with the H5, N1 and M2 cDNA clones. Thank-you to Dr. Robert Webster at the St. Jude’s Childrens’ Research Hospital in Memphis, TN for supplying the monoclonal anti-HA antibodies. Thank you to Dr. Rob Kotin for supplying us with the anti-VP39 antibody. Thank-you to Dan-hui Yang, a previous post-doctoral fellow in the Krell and Nagy labs for supplying us with the FAdV-HA viruses and serum from vaccinated chickens. Thank you to OGS, OMAFRA and NSERC for funding this research.

The members of the Krell lab have been indispensible to the completion of this research. Thank you to Jeffrey Hodgson for training me in the lab and for your continued support and plentiful guidance throughout this research. Thank you to James Ackford, an undergraduate project student for your contribution to the NA over-expression work. Thank you to Yang, Dave, Mike, J.D. and Guozhong for your friendship, advice and encouragement.

Without the support of my family and friends, the completion of this Masters would not have been possible. Thank you to my parents, Anne and Morris, brother Cameron, sister Lauren, and brother-in-law Ned for your love and support. Finally, I would like to thank my partner and best friend Nathan for your continued emotional support throughout the past two years. It is such a comfort to know that no matter what, you are always in my corner.
# TABLE OF CONTENTS

Title Page ................................................................. i
Abstract ........................................................................ ii
Acknowledgements ...................................................... iii
Table of Contents ........................................................ iv
List of Figures ............................................................... vii
List of Tables ............................................................... ix
List of Abbreviations .................................................... x
List of Virus and Protein Abbreviations ......................... xii

Chapter 1: Review of the Literature .................................. 1
  1.1 Influenza and influenza virus ................................... 1
  1.2 Hemagglutinin and neuraminidase .............................. 5
  1.3 Evolution of influenza viruses and avian influenza virus .... 8
  1.4 HPAI control strategies in humans ................................. 11
  1.5 HPAI control strategies in poultry ................................ 14
  1.6 Baculoviruses as expression vectors .............................. 15
  1.7 Baculovirus expression, overexpression and display ........ 18
  1.8 Applications of baculoviral foreign gene expression to influenza virus research .... 23
  1.9 Research objectives and experimental design .................. 27

Chapter 2: Materials and Methods .................................... 30
  2.1 Cells and virus ........................................................ 30
  2.1.1 Insect cell culture ............................................... 30
  2.1.2 Influenza virus, HA and NA clones .......................... 30
  2.2 General DNA manipulation ......................................... 30
  2.2.1 Bacterial cultures and plasmid isolation ....................... 30
  2.2.2 Restriction digestions and PCR ................................. 31
  2.2.3 Plasmid construction and cell transformation ............... 32
  2.3 Generation of recombinant baculovirus constructs .......... 32
  2.3.1 Removal of histidine (6-His) tag from pFastbacB .......... 32
  2.3.2 Generation of ΔHisFastBacBHAFLAG ....................... 35
  2.3.3 Generation of ΔHisFastBacBHA:VP39FLAG and ΔHisFastBacBVP39:HAFLAG (VP39 fusion constructs) .......... 35
  2.3.4 Generation of ΔHisFastBacBHA:GP64FLAG ................. 37
  2.3.5 Generation of ΔHisPFastbacBNAHA ......................... 38
Chapter 3: Results

3.1 Generation of Recombinant viruses ..........................................................50
  3.1.1 Development of the baculovirus recombinant HA viruses ......................52
  3.1.2 Viral growth kinetics ..............................................................................52

3.2 Expression of HA by three different methods as measured using anti-FLAG antibody
  3.2.1 HAFLAG overexpression .....................................................................52
  3.2.2 HAFLAG expression in Sf21 cells ..........................................................54
  3.2.3 AcHAFLAG expression in Hi-Five™ cells ............................................53
  3.2.4 Comparison of HAFLAG recovery between NP40 and RIPA lysis buffers for ELISA .................................................................58
  3.2.5 Nature of 65 and 74 kDa bands: serum-containing versus serum-free media...60
  3.2.6 HAFLAG expression in Sf21 cells in the presence or absence of tunicamycin ...60
  3.2.7 AcGP64:HAFLAG expression ................................................................63
  3.2.8 AcHA:VP39FLAG and AcVP39:HAFLAG expression .................................65
  3.2.9 Relative levels of HA expression between all HA-expressing baculoviruses ...68

3.3 Recognition of HA expressed by recombinant viruses by antibodies against HA ....71
  3.3.1 Detection of HAFLAG with a commercial polyclonal, mono-specific (H5) HA antibody ..................................................................................................72
  3.3.2 Detection of HA from recombinant HA-display and HAFLAG viruses using a
commercial polyclonal, monospecific anti-HA antibody ........................................70
3.3.3 Detection of HA from all recombinant baculoviruses with monoclonal anti H5
HA antibodies ........................................................................................................72
3.3.4 Detection of HA in Sf21 cells infected with each recombinant HA-expressing
baculovirus using polyclonal anti-serum to H5N1 virus ......................................77
3.3.5 Testing of serum from FAdV-HA vaccinated birds using HA\textsubscript{FLAG} as antigen .79

3.4 Biological activity of HA expressed in the insect cell ........................................79
  3.4.1 Hemagglutination assays ............................................................................79
  3.4.2 Hemadsorption assays .............................................................................83
  3.4.3 Formation of syncytia (CPE) in Sf21 cells infected with recombinant HA-
  expressing baculoviruses .....................................................................................87

3.5 Neuraminidase expression ..............................................................................89
  3.5.1 Neuraminidase overexpression ..................................................................89
  3.5.2 Neuraminidase assay ................................................................................89

Chapter 4: Discussion ..............................................................................................93
  4.1 Characterization of expressed HAs using an anti-FLAG antibody ...............94
  4.2 Characterization of expressed HAs using anti-HA antibodies ..................104
  4.3 Biological activity of recombinant HAs .........................................................108
  4.4 Neuraminidase expression ..........................................................................110
  4.5 Summary and future directions ...................................................................111

References .............................................................................................................115

Appendices ...........................................................................................................127
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Influenza virus virion</td>
<td>4</td>
</tr>
<tr>
<td>1.2</td>
<td>Hemagglutinin protein and schematic</td>
<td>7</td>
</tr>
<tr>
<td>1.3</td>
<td>Geographic distribution of H5N1 in humans</td>
<td>12</td>
</tr>
<tr>
<td>1.4</td>
<td>Baculovirus budded virion</td>
<td>17</td>
</tr>
<tr>
<td>1.5</td>
<td>Flowchart of recombinant baculovirus generation using Bac-to-Bac (Invitrogen)</td>
<td>20</td>
</tr>
<tr>
<td>1.6</td>
<td>Schematic of GP64 and VP39 display</td>
<td>24</td>
</tr>
<tr>
<td>2.1</td>
<td>Schematic representation of ΔHisPFastbacB</td>
<td>33</td>
</tr>
<tr>
<td>2.2</td>
<td>Schematic representation of all recombinant baculoviruses generated</td>
<td>49</td>
</tr>
<tr>
<td>3.1</td>
<td>Schematic representation of recombinant HA-expressing baculoviruses generated</td>
<td>51</td>
</tr>
<tr>
<td>3.2</td>
<td>Virus growth kinetics</td>
<td>53</td>
</tr>
<tr>
<td>3.3</td>
<td>Western blot of AcHAFLAG transfected Sf21 cells using anti-FLAG antibody</td>
<td>55</td>
</tr>
<tr>
<td>3.4</td>
<td>Western blot of AcHAFLAG infected Sf21 and Hi-Five™ cells using anti-FLAG antibody</td>
<td>56</td>
</tr>
<tr>
<td>3.5</td>
<td>Western Immunoblot of AcHAFLAG infected Hi-Five™ cells, comparing NP40 and RIPA lysis buffers using anti-FLAG antibody</td>
<td>59</td>
</tr>
<tr>
<td>3.6</td>
<td>Western Immunoblot comparing serum free and serum containing medium in AcHAFLAG infected Hi-Five™ cells using anti-FLAG antibody</td>
<td>61</td>
</tr>
<tr>
<td>3.7</td>
<td>Western Immunoblot comparing AcHAFLAG infected Sf21 cells in presence or absence of tunicamycin using anti-FLAG antibody</td>
<td>62</td>
</tr>
</tbody>
</table>
3.8 Western Immunoblot of AcHA:GP64FLAG infected Sf21 and Hi-Five™ cells using anti-FLAG antibody  
3.9 Western Immunoblot of AcHA:VP39FLAG and AcVP39:HAFLAG infected Sf21 and Hi-Five™ cells using anti-FLAG antibody  
3.10 Western Immunoblot comparing all HA-expressing baculoviruses using anti-FLAG antibody  
3.11 Western Immunoblot comparing HA expression in budded virions of all HA-expressing viruses using anti-FLAG antibody  
3.12 Western Immunoblot of AcHAFLAG infected Sf21 cells using polyclonal anti H5 monospecific antibody #3425  
3.13 Western Immunoblot of Sf21 cells infected with all HA-expressing viruses, using the polyclonal anti H5 monospecific antibody #3425  
3.14 Western Immunoblot of Sf21 cells infected with all HA-expressing viruses, using a monoclonal anti-H5 HA antibody  
3.15 Western Immunoblot of Sf21 cells infected with all HA-expressing viruses, using a polyclonal H5 chicken serum  
3.16 Western Immunoblot of Sf21 cells infected with AcHAFLAG using serum from chickens vaccinated with FAdV-HAᵣ as primary antibody  
3.17 Hemagglutination assays  
3.18 Hemadsorption assays  
3.19 Syncytia formation in AcHAFLAG and AcHA:GP64FLAG infected cells  
3.20 Western Blot of AcNAHA infected Sf21 cells  
3.21 Biological activity of NA
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>PCR conditions</td>
<td>31</td>
</tr>
<tr>
<td>2.2</td>
<td>List of primers used for cloning</td>
<td>34</td>
</tr>
<tr>
<td>2.3</td>
<td>List of primers used for sequencing</td>
<td>36</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

AI  avian influenza
bp  base pairs
BEVS baculovirus expression vector systems
cDNA complementary DNA
CP  circumsporozoite protein
CPE  cytopathic effect
DIVA differentiation of infected from vaccinated
DNA  deoxyribonucleic acid
DNA pol  DNA polymerase
dsDNA double stranded DNA
ELISA enzyme-linked immunosorbent assay
ER  endoplasmic reticulum
FAdV  fowl adenovirus
FBS  fetal bovine serum
GOI  gene of interest
GST  glutathione-S-transferase
GP64  glycoprotein 64
HA  hemaglutinin
His  histidine-tag
HPAI highly pathogenic avian influenza
h.p.i. hours post infection
h.p.t. hours post transfection
Kb  kilobases
kDa kilodaltons
M1  matrix 1 protein
M2 matrix 2 ion channel
LPAI low pathogenic avian influenza
LB  Luria Bertanni
min minute(s)
MOI  multiplicity of infection
mRNA messenger ribonucleic acid
NA  neuraminidase
NP  nucleoprotein
NS1  non-structural protein 1
NS2  non-structural protein 2
ODV  occlusion derived virus
ORF open reading frame
ori  origin of DNA replication
PBS  phosphate buffered saline
PCR polymerase chain reaction
Pfu  plaque-forming units
PHAC Public Health Agency of Canada
pi  post-infection
polh  polyhedrin promoter
rBV recombinant budded virus
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpm</td>
<td>rotations per minute</td>
</tr>
<tr>
<td>SAα2,3gal</td>
<td>sialic acid alpha 2, 3 galactose receptor</td>
</tr>
<tr>
<td>SAα2,6gal</td>
<td>sialic acid alpha 2,6 galactose receptor</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SP</td>
<td>signal peptide</td>
</tr>
<tr>
<td>TBE</td>
<td>tris borate EDTA</td>
</tr>
<tr>
<td>TIV</td>
<td>trivalent inactivated vaccine</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VLP</td>
<td>virus-like particle</td>
</tr>
<tr>
<td>VP39</td>
<td>viral protein 39</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>wt</td>
<td>wild-type</td>
</tr>
</tbody>
</table>
# VIRUS AND PROTEIN NAMES

<table>
<thead>
<tr>
<th>Virus Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcMNPV</td>
<td>Wild-type <em>Autographa californa</em> multienucleopolyhervirus</td>
</tr>
<tr>
<td>AcΔCC</td>
<td>Recombinant AcMNPV with the chitinase and cathepsin ORFs removed</td>
</tr>
<tr>
<td>AcCHIA_FLAG</td>
<td>Recombinant AcMNPV ΔCC expressing chitinase and a FLAG epitope tag</td>
</tr>
<tr>
<td>AcCHIA(ASD)_FLAG</td>
<td>Recombinant AcMNPV ΔCC expressing the active site domain of chitinase and a FLAG epitope tag</td>
</tr>
<tr>
<td>FAdV</td>
<td>Fowl adenovirus</td>
</tr>
<tr>
<td>FAdV-9Δ4</td>
<td>Fowl adenovirus with the 9-4 region removed</td>
</tr>
<tr>
<td>FAdV-HA</td>
<td>Fowl adenovirus 9Δ4 with HA cloned either in the left (L) or right (R) orientation</td>
</tr>
<tr>
<td>AcHA_FLAG</td>
<td>Recombinant AcMNPV ΔCC expressing FLAG-tagged HA</td>
</tr>
<tr>
<td>AcHA:GP64_FLAG</td>
<td>Recombinant AcMNPV ΔCC expressing FLAG-tagged HA:GP64 fusion</td>
</tr>
<tr>
<td>AcHA:VP39_FLAG</td>
<td>Recombinant AcMNPV ΔCC expressing FLAG-tagged HA:VP39 fusion</td>
</tr>
<tr>
<td>AcVP39:HA_FLAG</td>
<td>Recombinant AcMNPV ΔCC expressing FLAG-tagged VP39:HA fusion protein</td>
</tr>
<tr>
<td>CHIA_FLAG</td>
<td>FLAG tagged chitinase protein</td>
</tr>
<tr>
<td>CHIA(ASD)_FLAG</td>
<td>FLAG tagged active site domain of chitinase protein</td>
</tr>
<tr>
<td>HA_FLAG</td>
<td>FLAG tagged HA</td>
</tr>
<tr>
<td>HA:GP64_FLAG</td>
<td>FLAG tagged HA:GP64 fusion protein</td>
</tr>
<tr>
<td>Description</td>
<td>Description</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>HA:VP39\textsubscript{FLAG}</td>
<td>FLAG tagged HA:VP39 fusion protein</td>
</tr>
<tr>
<td>AcVP39:HA\textsubscript{FLAG}</td>
<td>FLAG tagged VP39:HA fusion protein</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 Influenza and influenza viruses

Influenza (flu) is a highly infectious, acute respiratory illness caused by influenza viruses, and is associated with significant mortality and morbidity worldwide (Simonsen et al., 1998). Influenza epidemics occur both annually and seasonally in most parts of the world, with peak activity reported between December and March in northern temperate climates, and throughout the year in tropical climates (Cox and Subbarao, 2003). In humans, influenza virus infection is characterized by a broad collection of symptoms, including: extreme fatigue, headache, fever, general achiness, sore throat, and occasionally dry cough or runny nose. In susceptible individuals, such as the elderly, the very young, or those with compromised immunity, serious pulmonary, neurological, or cardiovascular complications can arise in association with influenza virus infection (Cox and Subbarao, 2003). Cases of bacterial pneumonia (Rothberg and Haessler, 2010), myocarditis (Ison et al., 2005) and encephalopathy (Studahl, 2003) have all been documented in patients admitted to hospital and diagnosed with primary influenza virus infection. Additionally, this disease can exacerbate underlying or pre-existing medical conditions such as asthma, or atherosclerosis (Rothberg and Haessler, 2010).

The symptoms of influenza are often ambiguously described, and since complications associated with this illness can be broad, influenza-related deaths tend to be underreported. As a result, the true burden of this disease on the Canadian population has most likely been underestimated. For instance, in 2011 there were approximately 18,000 laboratory-confirmed cases of influenza virus in Canada (Public Health Agency of Canada, 2012), representing 0.5% of the Canadian population, of 34 million (World Bank, 2010). However, it has been estimated that up to 20% of the population (roughly 6.8 million Canadians) may become infected during
any given flu season (Parkins et al., 2009). These numbers, which are significantly greater than what is actually reported in hospitals, are likely indicative of the seriousness of this disease, emphasizing the importance of prevention. Further, while the indirect implications of influenza on populations may be less overtly obvious, they are in fact consequential. For instance, it has been noted that during a pandemic, influenza-related absenteeism and reduced household consumption can result in a 10% loss of gross domestic product (Verikios et al., 2010), making it clear that influenza virus infection can have far-reaching and negative implications.

Currently, the most efficacious way to prevent influenza virus infection and its potentially detrimental complications is through vaccination (Fiore et al., 2010). In addition, two classes of antiviral drugs, including neuraminidase inhibitors (such as Zanamivir) and ion channel (M2) blockers (such as Rimantadine) have been approved for use against influenza virus (Fiore et al., 2010). Unfortunately, antiviral drugs are beneficial only when administered 24 to 48 hours post-infection, and therefore are often rendered ineffective (Fiore et al., 2010). Further, there is growing concern that the virus population is becoming resistant to anti-viral drugs. Due to their several limitations, antiviral drugs are generally prescribed only for individuals that are considered to be at high-risk for contracting influenza virus, are in direct contact with farm animals which may carry the virus, or in individuals where vaccination is contraindicated (e.g. egg allergy).

Influenza viruses belong to the family *Orthomyxoviridae*, which comprises five genera: *Influenzavirus A, Influenzavirus B, Influenzavirus C, Isavirus and Thogotovirus* (9th International Committee on the Taxonomy of Viruses (ICTV) report, 2011). The influenza virus genome is segmented and consists of eight linear, single-stranded ribonucleic acids (RNA) of negative polarity (Fig 1.1). The enveloped influenza viral particles are comprised of ten structural
proteins, including hemagglutinin (HA) and neuraminidase (NA) (Samji, 2009). Two of the eight viral transcripts are alternatively spliced and encode four polypeptides while the other six encode six polypeptides. Influenza virus particles, which range from 80-120 nm in length, are spherical or filamentous in nature, and this distinctive morphology is mediated in part by interactions of the well conserved cytoplasmic tails of the viral proteins HA and NA with internal viral constituents such as the matrix 1 protein (M1) (Jin et al., 1997). Without these tails, budding of influenza virus appears to be inefficient (Jin et al., 1997). HA and NA are abundant surface glycoproteins which also mediate critical interactions between virus and host cell (Samji, 2009).

The three genera, *Influenza virus A*, *Influenza virus B*, and *Influenza virus C*, comprise type species, *Influenza A virus*, *Influenza B virus* and *Influenza C virus*, respectively. While viruses of all three species are capable of infecting humans, influenza viruses A and B are primarily responsible for causing epidemics. Historically, pandemics are caused exclusively by influenza A virus. The nomenclature of influenza A virus is defined by the antigenic variation of the surface glycoproteins HA and NA (Bouvier and Palese, 2008). There are at least sixteen subtypes of HA (H1-16) and nine subtypes of NA (N1-9), but only H1- H3, as well as N1 and N2 have been identified in human influenza virus epidemics (Bouvier and Palese, 2008).
Figure 1.1 The influenza virion. Eight RNA segments code for ten viral proteins via alternative splicing. HA, NA and M2 are the surface glycoproteins. Image from http://www.virology.ws/2009/04/30/structure-of-influenza-virus/.
1.2 Hemagglutinin and neuraminidase

The influenza virus major ~64 kilodalton (kDa) glycoprotein HA, is a trimeric fusion protein and is critical for attachment to sialic acid receptors on the host cell. HA$_0$ represents the inactive form of this protein. Being an integral membrane protein, HA is translated and modified through the endoplasmic reticulum (ER), and is subsequently transported through the Golgi apparatus to the cell membrane. During this translocation, HA$_0$ undergoes several post-translational modifications, including: formation of disulfide bonds, whose quantity varies among different HAs but which all function to stabilize the protein (Chreighton, 1988, and Segal et al., 1992), addition of N-glycosidic oligosaccharide side chains (Keil et al., 1985), acylation (Schmidt, 1982), and proteolytic cleavage into two active subunits, amino-terminal HA$_1$ and carboxy-terminal HA$_2$ (Huang et al., 1981, Klenk et al., 1975, and Lazarowitz et al., 1975).

Proteolytic cleavage of HA$_0$ is particularly important for influenza virus pathogenicity and apathogenic and pathogenic strains tend to be activated by different types of proteases (Stieneke-Grober et al., 1992). Cleavage of HA$_0$ in apathogenic strains is facilitated by non-ubiquitous proteases and therefore causes only localized infection in certain types of cells (Stieneke-Grober et al., 1992). Conversely, HA found in pathogenic strains of influenza virus undergoes cleavage by subtilisin-like endoproteases, which are ubiquitous and therefore present in numerous cell types, allowing for systemic infection rather than localized infection. Specifically, HAs from pathogenic strains are cleaved by furin proteases, a substituent of the subtilisin-like protease family (Stieneke-Grober et al., 1992). HA cleavability is largely determined by the amino acid sequence found at the cleavage site. Apathogenic strains have one arginine at this site while pathogenic strains have numerous lysines and arginines, with a specific
consensus sequence of R-X-K/R-R required for cleavage activation at these sites (Vey et al., 1992).

The biologically active, folded HA protein is composed of a globular head region that contains residues exclusively from the HA\textsubscript{1} subunit, as well as the sialic acid binding site and the major antibody binding sites (Wilson, et al., 1981). There is also a fibrous stem domain that contains residues from HA\textsubscript{2}, which comprises the transmembrane domain and membrane fusion peptide (Wilson et al., 1981) (Figure 1.2).

NA is a tetrameric rod-shaped spike protein on the surface of the influenza virion. NA exhibits sialidase activity, functioning to remove sialic acid residues from virus progeny and host cells, facilitating release and spread of new virus (Gubareva and Hayden, 2006). NA is a type II transmembrane protein, and therefore is N-terminally located inside the cell membrane, with a short cytoplasmic tail followed by the transmembrane domain, which allows for translocation to the membrane of the infected cell. NA also has a carboxy-terminal catalytic site, which is subjected to post-translational glycosylation at various sites (Colman et al., 1983; Markoff et al., 1984). Although NA is about five times less abundant on the surface of the influenza virion than HA, it is still the second immune-dominant influenza virus protein. Though antibodies against NA are not, strictly speaking, neutralizing, they do confer protection against the virus \textit{in vivo} (Wu et al., 2010).

Matrix 2 protein (M2), the third immunogenic influenza virus protein, is an integral membrane protein (Lamb et al., 1985), and ion channel which is present in low quantities on the surface of the virion (Pinto et al., 1992). Activation of the channel by histidine residues on the transmembrane domain of M2 occurs after virus receptor-mediated endocytosis,
Figure 1.2 A) 3D structure of globular HA protein. HA₀ undergoes proteolytic cleavage into 328 amino acid HA₁ (green) and 222 amino acid HA₂ subunits (cyan) (structure from Rachakonda 2006). B) Schematic representation of the HA protein. HA₀ has an amino terminal signal peptide (SP) and a carboxy-terminal 38 aa transmembrane domain (TM) followed by a short cytoplasmic domain (CD). HA₁ has several glycosylation (G) sites.
when the M2 ion channel encounters the low pH of the endosome inside the host cell, eventually leading to viral uncoating. This occurs via the dissociation of the M1 from the ribonucleoprotein (RNP), a critical step in the infection process (Wang et al., 1995). RNP is formed by binding of the viral nucleoprotein (NP) to the viral RNAs, and this bound complex is the active particle responsible for viral transcription and replication (Klumpp et al., 1997).

Other viral proteins include non-structural proteins 1 and 2 (NS1 and NS2), involved in viral processing (Lin et al., 2007) and the host anti-viral response, and the viral RNA polymerase subunits PB1, PB2, and PA.

1.3 Evolution of influenza viruses and avian influenza virus

Influenza virus evolution occurs through two distinct processes, antigenic drift and antigenic shift. Antigenic drift represents mutations leading to slight antigenic changes incurred by the circulating HA or NA subtype within a population over a period of time (Subbarao, 2006). Antigenic drift does not cause the HA subtype to change, but is sufficient to necessitate a newly designed seasonal influenza vaccine each year because antibodies can no longer neutralize, or can only weakly neutralize, the mutated form of the virus (Subbarao, 2006). In contrast, antigenic shift represents a significant change in which a circulating HA or NA subtype is exchanged either for a different one, or for the same subtype from a different virus. This creates a mosaic virus which then may be introduced into an immunologically naive population.

Antigenic shift occurs when there is a genetic reassortment of different influenza viruses within the same cell in a common host, infected by two different HA (or NA) subtype viruses. The most recent example of this type of shift is that which resulted in the 2009 H1N1 pandemic, which originated and reassorted in swine (Vijaykrishna et al., 2010). This novel virus was the
result of a quadruple reassortment event in which classical swine H1N1, North American avian
H1N1, human seasonal H3N2 and Eurasian avian-like swine H1N1 viruses all reassorted in
swine, which acted as a mixing vessel for the three, and subsequently infected humans
(Schnitzler and Schnitzler, 2009). While different influenza virus proteins came from these
various sources, the HA was linked to classical North American swine and the NA originated
from Eurasian avian-like swine viruses (Schnitzler and Schnitzler, 2009).

Influenza virus is endemic in many bird species, including migratory birds and
waterfowl. While generally asymptomatic in wild birds, which serve as a natural reservoir for the
virus, some influenza viruses can be extremely pathogenic in poultry. There are two distinctive
pathotypes of avian influenza (AI) viruses based on their ability to cause disease in chickens
(Alexander, 2000; Booy et al., 2006): low pathogenicity AI (LPAI) or high pathogenicity AI
(HPAI). LPAI viruses can occur in domestic chickens and are not typically lethal (Subbaroao,
2006), while HPAI viruses can cause severe disease. The majority of avian influenza viruses are
LPAI viruses, and are comprised of all HA subtypes. In contrast, HPAI is extremely lethal and to
date has traditionally involved only the H5 and H7 subtype viruses (Subbaroao, 2006).

A HPAI virus H5N1, which emerged in domestic poultry in the late 1990’s in Asia,
carries significant pandemic potential due to its observed capability for avian to human
transmission (Webster, 2006). This virus originated in geese in the Guandong region of China in
1996, and was the initial source of influenza virus which later infected a child in 1997 and
resulted in the death of eighteen other individuals in Hong Kong (Lignon, 2005). Further, despite
the culling of infected poultry following the outbreak, this virus still circulates in wild geese and
ducks within China, and has since been responsible for other H5N1 human infections (Webster,
2006). Normally, avian and human influenza viruses preferentially bind to receptors with
saccharides that terminate at sialic-acid α2, 3 galactose (SAα2,3gal), and SAα2,6gal, respectively (Caroll et al., 1981). For a HPAI H5N1 virus to be directly transmitted from birds to humans, it must have undergone a mutation which allowed for recognition of SAα2,6gal receptors. However, because the virus lacks sustained human-to-human transmission, recognition of a SAα2,6gal receptor must be limited (Yamada et al., 2006). In order for this HPAI H5N1 virus to cause a human pandemic, it must mutate further to recognize SAα2,6gal with greater efficiency than it currently does (Yamada et al., 2006).

In addition to the obvious threat HPAI poses to human health, the detrimental effects of this virus on the poultry industry are also significant. HPAI is currently present in poultry farms all over the world, particularly in developing countries. In China, HPAI H5N1 is more widespread than in other parts of the world, and this has been attributed to a number of factors. First, 70% of poultry production occurs in backyard flocks, meaning that many humans tend to be in close proximity to live chickens daily (Peiris et al., 2007). Second, live poultry markets are customary in China, and often include ducks and other waterfowl, which can be asymptomatic and shed the virus for up to 17 days (Peiris et al., 2007). Once HPAI H5N1 has been introduced to a live poultry market, eradicating the outbreak becomes nearly impossible (Peiris et al., 2007). Finally, many poultry farms are located in remote areas of China, where access to veterinary care may be limited, so improper or non-identification of HPAI H5N1 virus results in endemics.

Chickens inoculated with HPAI H5N1 virus have a mortality rate of up to 75% in a laboratory setting, however flock mortality can be up to 100% following natural infection. Thus the impact of HPAI on the economies of countries that strongly rely on poultry production can be devastating (Swayne and Suarez, 2000 and Webster et al., 1992). Consequently there is a substantial need for a reliable and universal vaccine-based control strategy in poultry, along with
a strong commitment from political leaders, veterinarians and public health officials to ensure successful implementation and surveillance of vaccinated and infected birds.

1.4 HPAI control strategies in humans

In Canada, human influenza virus surveillance is by the Public Health Agency of Canada (PHAC) and in coordination with the World Health Organization (WHO). The PHAC collects data on confirmed influenza cases, school absenteeism, and circulating influenza viruses, and reports weekly on activity within Canada. This information is provided to the WHO and is used in part to develop official recommendations for the components of the next seasonal vaccine. The currently recommended and used vaccine is a trivalent inactivated vaccine (TIV), which is produced in chicken embryos. In general, two influenza A viruses and one influenza B virus are present in the seasonal vaccine. For instance, in February 2012, the WHO, based on data collected from September 2011 to January 2012, recommended that the three candidate influenza viruses for the 2012/2013 northern hemisphere vaccine be: A/California/7/2009 (H1N1) pdm-09-like virus, A/Victoria/361/2011(H3N1)-like virus, and B/Wisconsin/1/2010-like virus (WHO, 2012). The names of human influenza viruses are based on the genus, geographic location of origin, isolate number, year of origin and subtype, respectively. For instance, the official name for one isolate of the pandemic influenza H1N1 virus of 2009 is: A/Mexico/4108/2009 (H1N1) (WHO, 2010).

Import of animals and animal products which may have been exposed to influenza virus is strictly regulated. In Canada, this responsibility falls primarily under the Canadian Food Inspection Agency (CFIA), and the Canadian Border Control Services (CBCS), which carry out surveillance via the Canadian Notifiable Avian Influenza Surveillance System (CanNAISS).
Figure 1.3 Geographic distribution of human cases of H5N1 AI (WHO, 2011) Web address: http://gamapserver.who.int/mapLibrary/Files/Maps/Global_H5N1inHumanCUMULATIVE_FIMS_20110316.png
This surveillance system carries out a number of activities, including: wild bird surveillance, passive and targeted surveillance when clinical signs of avian influenza are reported, pre-slaughter surveillance in commercial poultry, hatchery supply flock surveillance, and voluntary surveillance in the poultry genetics export sector (CFIA, Government of Canada, 2012). The Canadian Government also follows guidelines established by the WHO which state that those AI viruses with high pathogenicity, as well as all AI viruses of the H5 and H7 subtypes, regardless of pathogenicity, must be reported to the Canadian Food Inspection Agency (CFIA). Moreover, import of chickens and chicken products from countries where avian influenza virus outbreaks are frequent or widespread is strictly prohibited. Currently, for example, import of birds from China, Bangladesh, Egypt, India, Indonesia and Vietnam is banned due to the strong presence of HPAI AI in these areas.

Though current vaccine strategies in humans are sufficient for managing seasonal influenza virus, they do have some distinct limitations. For instance, the production of virus in chicken embryos is rather slow, and typically takes anywhere from 7 to 11 months (Booy et al., 2006) to produce and scale up, and therefore would be insufficient to respond in an emergency situation, such as a pandemic, or in the event of a new or late-appearing strain. Additionally, vaccination of an entire population with an egg-based vaccine is unlikely, since those with egg allergies and other sensitivities may be less inclined to be vaccinated even with the availability of non-egg derived vaccines. Further, a problem that applies to H5N1 avian influenza virus specifically is that the virus kills chicken embryos, and therefore less vaccine can be produced, making the production process more costly. This in itself poses significant problems when considering that HPAI H5N1 carries pandemic potential, as sufficient and reliable vaccine production would be an absolute necessity in an emergency situation. Additionally, the loss of
embryos due to highly virulent H5N1 in the development process would be extremely costly. Clearly, these inadequacies with the traditional vaccine necessitate the development of a new vaccination strategy that will be sufficient to handle H5N1 avian influenza virus and overcome some of these current challenges.

1.5 HPAI control in poultry

In conjunction with an effective human HPAI H5N1 influenza virus vaccine, immunization of poultry represents an important strategy for HPAI H5N1 control and prevention. Currently two vaccines expressing HA, one which is an inactivated whole virus vaccine and the other which is a recombinant fowlpox virus vectored vaccine (Swayne et al., 2000), have been approved for vaccination of poultry (Subbaroao, 2006). While this practice has not yet been officially approved as a control mechanism in Canada (CFIA, 2009), other countries including Italy (Capua et al., 2003), the USA (Halvorsen et al., 2002), Pakistan (Naeem et al., 2006) and Mexico (Villareal and Flores, 2003) have successfully vaccinated against circulating LPAI viruses, suggesting that vaccination of poultry against HPAI viruses may also be promising.

In Canada and elsewhere, there are a few key barriers to the successful implementation of HPAI H5N1 vaccination in poultry. First, unless a dependable strategy is implemented to differentiate vaccinated from infected animals (DIVA), it is difficult to ensure that antibodies present in chickens are due to vaccination rather than a new or pre-existing influenza virus infection. Because live birds showing serological evidence of influenza virus infection cannot be exported or imported according to Canadian law (CFIA 2012), a reliable DIVA is imperative in order to execute successful vaccination of poultry. While several candidate vectors for antigen
have been established for the DIVA approach, all require diligent serological monitoring and therefore can be both laborious and expensive.

Enzyme-linked immunosorbent assays (ELISAs) may be promising in the development of a DIVA. An ELISA is a rapid serological diagnostic test which entails exposing a serum sample to antigen and visually observing, using an alkaline phosphotase (most commonly) conjugated secondary antibody, whether that serum contains antibodies against it. ELISAs are often employed as an initial test in the diagnoses of various diseases. For example, ELISAs are used as an initial test in determining whether an individual is producing anti-HIV antibodies (Tamashiro et al., 1993).

Another barrier associated with the successful execution of poultry vaccination is that due to its bio-safety containment requirements, live HPAI H5N1 virus cannot be used as a source of HA in many laboratories. As a result, the only way to monitor antibody response to an H5N1 virus vaccine is to use an alternative source for H5 HA. A potential expression vector to produce large amounts of biologically active HA, safely and robustly, is the Baculovirus Expression Vectors System (BEVS).

1.6 Baculoviruses as expression vectors

Insect baculoviruses provide a potential effective vector for the development of a subunit vaccine or diagnostic antigen based on HA or NA. Baculoviruses, belonging to the *Baculoviridae* family, are a large group of covalently closed, circular, double–stranded DNA viruses with genomes ranging from 88-153 kb (Blissard et al., 1990). Baculoviruses infect at least six hundred insect species (Martignoni et al., 1986) and are classified into four genera: *Alphabaculovirus, Betabaculovirus, Deltabaculovirus* and *Gammabaculovirus* (9th International
Committee on the Taxonomy of Viruses (ICTV) report, 2011). Baculoviruses have two distinct phenotypes, the occlusion derived virus (ODV), required for environmental dissemination of the virus, and the budded virion (BV), necessary for systemic spread of the virus in the infected insect (budded virion depicted in Fig 1.4). The genus *Alphabaculovirus* is comprised of all lepidopteran-specific nucleopolyhedroviruses and notably, contains the prototypic and most characterized baculovirus, *Autographica californica* multiple nucleopolyhedrovirus (AcMNPV), which is also the basis for most baculovirus expression vectors. Baculoviruses are attractive vectors for the expression of foreign proteins for a few key reasons. First, in comparison to other viral vectors, e.g. adenovirus, baculovirus is capable of incorporating extremely large DNA inserts. In fact, the successful development of a recombinant baculovirus containing a 38 kb DNA insert producing adenoviral proteins in mammalian cells has been demonstrated (Cheshenko et al., 2001). These authors predict that the upper packaging limit of baculovirus may even exceed 50 kb. Other properties which make baculoviruses excellent for use in expressing foreign proteins include the simplicity and speed in which recombinant baculoviruses can be generated, their ability to concurrently incorporate numerous genes, and their capacity to transduce a wide variety of cell types, including even mammalian cells (Hofmann et al., 1995; Condreay et al., 1999; Shoji et al., 1997). Further, because recombinant baculoviruses replicate in insect cells, post-translational modifications critical for the production of biologically active protein from eukaryotic sources still occur. In recombinant baculovirus-expressed HA, for example, when processed in insect cells, recombinant HA still undergoes proteolytic cleavage and glycosylation, and these cells produce properly folded, biologically active protein (Kuroda et al., 1986).
Figure 1.4 Schematic of baculovirus budded virion morphology (ExPASy Bioinformatics Resource Portal, www.expasy.org, 2012).
Baculoviruses have been used very successfully as vectors for foreign genes both in vitro and more recently, in vivo (Bai et al., 2008; Facciabene et al., 2004) particularly in the development of pharmaceuticals, gene therapies, diagnostic reagents and vaccines. The latter application represents one of the more popular uses of baculovirus expression vector systems, and several vaccines have been developed which use recombinant AcMNPV as a delivery vehicle for immunogenic proteins.

Examples of recombinant baculovirus-based vaccines are numerous. Some notable vaccines developed using baculovirus expression vector systems (BEVS) include anti-malaria vaccines, which use BEVS to express either *Plasmodium falciparum* circumsporozoite protein (CP) (Strauss et al., 2007), or *Plasmodium yoelli* merozoite surface protein (Yoshida et al., 2010), a SARS-like corona virus vaccine construct which expresses the immunogenic spike protein to elicit an immune response (Bai et al., 2008), a Newcastle disease virus (NDV) vaccine which expresses hemagglutinin-neuraminidase (Nagy et al., 1994) and a vaccine targeting porcine reproductive and respiratory syndrome virus, expressing the GP5 and M proteins (Wang et al., 2007) just to mention a few. Of current interest to human disease and cancer prevention is the human papillomavirus (HPV)-like-particle vaccine Cervarix (GlaxoSmithKline), produced in insect cells BEVS (Senger et al., 2009). Cervarix, which was recently approved for use in Canada, is the first-ever baculovirus-based vaccine to be commercialized. The fact that this type of vaccine has been approved by the designated governing bodies based on safety and efficacy studies further confirms baculovirus as a promising vaccine vector for foreign protein. Additionally, it promotes further research into large-scale production of foreign proteins as reagents or antigen in diagnostics, for a number of potential applications in research, healthcare, and industry.
1.7 Baculovirus vector systems, overexpression and display

AcMNPV was developed into a protein expression system in the early 1980’s (Smith, 1983), and since then, the precision and versatility with which baculoviral expression can be accomplished has only improved. The traditional method for development of baculovirus expression vectors depended on homologous recombination, and co-transfection of insect cells with both baculovirus and transfer plasmid DNA containing the gene of interest (Kost et al., 2005). This method, which was immensely laborious and generally yielded less than 0.1% recombinant viral progeny (Luckow et al., 1993), was later replaced by a bacmid expression system, Bac-to-Bac (Invitrogen) (Fig. 1.5), which was originally developed by Luckow et al. (1993). This bacmid system uses a baculovirus-containing transfer vector equipped with the very late polyhedrin and/or p10 promoter, gentamicin resistance gene, and two Tn7 elements which allow for localized transposition of the expression cassette into the baculovirus genome. The recombinant bacmid is harvested and transfected into Spodoptera frugiperda (Sf21) insect cells initiating an infection and resulting in the production of recombinant virions. The supernatant from the transfection is then collected as the P1 recombinant virus stock, and then can be amplified. Once the recombinant virus is amplified and titred, it can be used for experimental infections, and if desired, for the development of diagnostic reagent. In 1995, a novel system in which a baculovirus could display a foreign protein of interest on the GP64 baculovirus major envelope protein was established (Boublik et al., 1995) (Fig.1.6). GP64 is a trimeric 64 kDa protein whose function is to facilitate pH-dependent membrane fusion, and initiate viral entry. GP64, which is 512 amino acids, has an N-terminal signal peptide (which is part of a glycosylated N-terminal ectodomain), and a C terminal transmembrane domain followed by a small cytoplasmic domain (Kadlec et al, 2008). GP64 is packaged in dense peplomers (Monsma
Figure 1.5 Flowchart of generation of a recombinant baculovirus using the Bac-to-Bac System (Invitrogen).
et al., 1996) on the surface of the baculovirus virion, and can be neutralized by anti-GP64 antibodies, illustrating the importance of this protein in the infectivity of the virus. In studies where the AcMNPV GP64 protein was inactivated, cell-to-cell transmission of baculovirus was limited, suggesting that without GP64, the likelihood of a successful virus infection is greatly minimized (Monsma et al., 1996).

Because of its role in the infectivity of baculovirus, it has been proposed that fusion of a foreign protein or antigen directly to GP64 might provide more efficient expression and antibody detection. While GP64 display has not been extensively studied, the proof-of-principle study showed that two proteins, glutathione-S-transferase (GST), and HIV major envelope protein gp120, can successfully be displayed on the baculovirus surface (Boublik et al., 1995). Researchers suggest that this display system might be superior to protein overexpression because it facilitates the display of foreign protein directly on the surface of budded virus particles, while the immunogenic activity of the foreign protein remains intact (Boublik et al., 1995), ultimately allowing for use of the budded virions rather than cellular lysates or purified protein as a source of the antigen.

In a recent study, the human enterovirus VP1 major capsid protein was inserted between the GP64 signal peptide and ectodomain (Meng et al., 2011). Using the baculovirus expression system, the fused GP64 protein was then expressed under a novel promoter, the white spot syndrome virus immediate early 1 (ie1) promoter. This promoter is active in both insect and mammalian cells, allowing for recombinant protein expression of transducer bacmids in both Sf21 cells and mice (Meng et al., 2011). The use of this promoter in conjunction with GP64 display encourages application of this baculovirus expression method as an alternative vaccine delivery platform.
While GP64 surface display represented a novel achievement in the field of baculoviral expression, and has some clear advantages such as its ability to actively display immunogenic proteins of other viruses directly on its surface, it does have some limitations. First, there is some speculation that because the foreign protein may alter the 3D structure of GP64, the infectivity of the recombinant baculovirus might also be compromised (Kukkonen et al., 2003). Second, with GP64 display, the protein of interest can be fused to only the N-terminus of the protein because when it was fused to the C-terminus or incorporated into the middle of GP64, virus was recovered, but the fusion protein was not effectively expressed (Boublik et al., 1995). Being able to fuse the protein of interest to only GP64 could make expression of foreign protein more challenging depending on the properties of the fusion gene (Boublik et al., 1995).

The newest baculovirus display method, capsid (VP39) display, involves fusion of foreign protein directly to the baculovirus major capsid protein (Fig 1.6). VP39, the major capsid protein forming the protective protein shell which surrounds the genetic material, has a notably versatile structure that can accommodate fusions of a foreign peptide to both the N and C termini, making it a practical and promising new baculovirus display system, analogous to phage display. This system, which was developed in 2003 (Kukkonen et al., 2003), has not been extensively reported on in the literature. There are only two other groups known to have successfully fused foreign protein to the baculovirus capsid. The first group, Song et al. (2010) fused ZnO, an inorganic peptide complex to the baculovirus capsid and a high titre of recombinant virus was subsequently produced. While this paper substantiates the potential usefulness and effectiveness of capsid display, the field to which the method was applied was
Figure 1.6 Schematic representation of GP64 fusion and VP39 fusion, and depiction of where the fusion proteins should be located on the budded virion or nucleocapsid (figure adapted from Kukkonen et al., 2003). This schematic is not accurate.
chemical technology, rather than biological or medical technology. The second and more recent publication, which is more applicable to the field of molecular biology, describes the use of VP39 major capsid protein as a delivery vector for ovalbumin (OVA) in mice (Molinari et al., 2011). OVA was successfully fused to the carboxy terminus of the VP39 protein, and was shown to effectively access the MHC I pathway for presentation on CD8 cells (Molinari et al., 2011). This research established that baculovirus capsid can successfully display heterologous antigens that retain their biological activity in the cell (Molinari et al., 2011), demonstrating preliminary evidence that baculovirus capsid display is an effective form for baculovirus-based expression.

1.8 Applications of baculoviral foreign gene expression to influenza virus research

Because of the limitations to the current trivalent influenza virus vaccine and in light of the fact that another influenza virus pandemic in the 21st century is highly likely, several research entities are investigating more effective vaccine alternatives, for both humans and poultry. Further, because of the pathogenicity of HPAI H5N1 and its increasing distribution throughout the world, there is a renewed sense of urgency within the scientific and health communities to develop a pandemic prevention plan that includes vaccination of chickens. To that end, a number of alternative influenza virus vaccines are currently undergoing stage 1 clinical trials. For instance, the efficacy of a vaccine containing HA fused with a flagellin ligand from a toll-like-receptor (TLR) and expressed in E. coli (Huleatt et al., 2008) is currently being investigated by Vaxinnate Corp. Other examples include: an HA-containing human adenovirus-vectored vaccine produced by Vaxin (Toro et al., 2007), and a vaccine in which matrix 2 (M2) protein is linked to a Hepatitis B virus-derived virus-like-particle (VLP), a non-infectious particle that resembles an influenza virion but contains no genetic material (Fiers et al., 2009). An M2 protein vaccine may be advantageous because this protein tends to be highly conserved in both avian and
human influenza viruses and could provide protection against different subtypes (Fiers et al., 2009).

Recombinant baculoviruses have also been used in a variety of contexts to corroborate evidence that influenza virus proteins can successfully be expressed by baculoviruses and elicit an immune response in vivo. For instance, Tao et al. (2009) used an avian H5N1 virus isolate to develop a VLP HA, NA and M1-containing influenza virus vaccine, and after assessing this vaccine in vivo, using a mouse model, confirmed that it was indeed sufficient for conferring protection against H5N1 isolates (Tao et al., 2009). This finding indicates that the utilization of HA, NA and M1 proteins from an avian-source influenza virus for the development of a vaccine should not only confer resistance to that particular strain of influenza virus, but also against circulating human strains which carry the same HA and NA subtypes. Another study of a similar nature found that a recombinant baculovirus-based 2009 pandemic H1N1 VLP vaccine expressing the same three proteins as the one aforementioned, HA, NA and M1, conferred protective immunity in ferrets against challenge with H1N1 influenza virus (Pushko et al., 2005). These studies, in combination with research that indicates influenza virus VLPs are capable of conferring resistance to a broader array of antigenic isolates (Bright et al., 2007) make baculovirus-based VLPs an attractive option when considering the different HPAI H5N1 influenza viruses, particularly in a pandemic situation.

Other examples of influenza virus vaccines developed in recombinant baculoviruses include: an HA-expressing vaccine derived from HPAI H5N1 in baculovirus pseudotyped with vesicular stomatitis virus glycoprotein (Wu et al., 2009), a recombinant vaccine expressing HPAI H5N1 HA on the surface protein GP64 of the baculovirus BmNPV (Jin et al., 2008), and a recombinant vaccine expressing HPAI H5N1 HA on GP64 of AcMNPV (Tang et al., 2010).
Clearly, baculoviruses have been employed extensively as a vector for the development of a variety of influenza virus targeted vaccines. Interestingly, a baculovirus-based vaccine, Flublok, manufactured by Protein Sciences, has completed all necessary phases of testing and is currently seeking FDA approval. This vaccine, which is trivalent and recombinant, expresses HA as antigen from the three circulating seasonal strains (Cox et al., 2008), and researchers are optimistic that it could ultimately replace the current vaccine.

While the aforementioned research emphasizes the capacity of baculovirus to effectively package and display immunogenic influenza virus proteins, it fails to provide insight into the application of these recombinant baculoviruses for other relevant purposes. For instance, recombinant baculoviruses expressing HA might be a better diagnostic source than HPAI H5N1 to measure the immune response of an influenza virus vaccine using ELISAs.

Several avenues related to baculovirus expression have yet to be explored. For instance, very few publications exist in which baculovirus capsid display has been employed or studied, and it would be interesting to determine the usefulness of this new approach in expression of HPAI H5N1 influenza virus proteins. Additionally, a comparison of different baculovirus expression systems and their efficiency has not been conducted to date. If the advantages and disadvantages to protein overexpression, capsid display, and surface display could be determined in controlled experiments using the same foreign protein(s), then it might help identify the best baculovirus expression system for this purpose. Some ways in which these expression systems could be compared include their ability to incorporate foreign protein for use as antigen (e.g., their ability to elicit antibody response by a vaccine) and the amount of functional foreign protein actually produced. If further research helps to optimize baculovirus expression, the use of recombinant baculovirus as antigen in DIVA analysis or for vaccine development may be
deemed more efficient and reliable, and ultimately may result in the more widespread adoption of baculovirus-based diagnostic antigens for the incorporation into a pandemic preparedness strategy involving immunization of poultry against HPAI H5N1.

1.9 Research objectives and experimental design

In response to the need for the development of a robust and effective vaccine for poultry, Nagy et al. (unpublished) have generated prototype fowl adenovirus vectored HA-based vaccine (FAdV-HA). The recombinant vector employed for the development of this candidate vaccine was described extensively by Corredor and Nagy in 2010 (Corredor and Nagy, 2010). In order to make this vaccine marketable, there must be a rapid test to show that vaccinated chickens are actually producing anti-HA antibodies effectively. This test must be cost-effective, rapid and efficient. Due to the highly pathogenic and infectious nature of H5N1 virus, whole live virus is unsuitable and unsafe for use as antigen, and therefore, an alternative source of H5 HA is required. An ELISA using a heterologous source for antigen (i.e. antigen not produced in FAdV) may represent an effective way to test sera from chickens vaccinated with FAdV-HA.

The major objective of this research was to establish the most proficient vector for producing hemagglutinin (HA) from highly pathogenic avian influenza (HPAI) H5N1 virus through BEVS. To achieve this end, the research was subdivided into three objectives.

The first objective was to generate several H5N1 HA-expressing recombinant baculoviruses, using three expression strategies: protein overexpression, envelope (GP64) display, and capsid (VP39) display. The latter of the three is a newly developed approach, and the expression of HA via this method would represent a novel achievement in the field. The experimental basis for comparison of these expression systems was mainly through monitoring
HA expression levels in cell lysates infected with each recombinant baculovirus, using SDS-PAGE and Western immunoblot. Additionally, recombinant virus growth kinetics in the insect cell were compared to each other and wild-type AcMNPV. Finally, the level of HA activity in Sf21 insect cells infected with each recombinant baculovirus was compared by using hemagglutination and hemadsorption assays.

The second objective was to compare each recombinant BV as an alternative to H5N1 as a source of H5 HA antigen to detect anti-HA antibodies produced following vaccination of chickens with FAdV-HA. The experimental approach was to conduct SDS-PAGE and Western immunoblots with sera from chickens vaccinated with FAdV-HA as the primary antibody.

The final goal of this research was to generate a recombinant baculovirus expressing the immunogenic influenza virus protein neuraminidase (NA) as an alternative antigen to be eventually used in conjunction with recombinant baculoviruses expressing HA, with the ultimate goal of applying it to an ELISA-based DIVA. While the ELISA has yet to be developed, the principle for the experimental approach is dependent on the generation of a baculovirus-expressed NA. If two recombinant baculoviruses are used as antigen in two separate ELISAs conducted in tandem, serum from an H5N1 influenza virus infected chicken would be positive for both HA and NA. Conversely, if the chicken was vaccinated with our prototype FAdV-HA, then the chicken serum should be positive only in the HA but not NA based ELISAs.

It was hypothesized that envelope display is the most optimal expression system for HA, because unlike protein overexpression HA should be incorporated directly into the baculovirus virion, rather than just in the insect cells. Further, and unlike the major capsid protein, the major envelope protein is expressed directly on the surface of the virion and cells, and is processed
through the ER, suggesting that HA should undergo its regular post-translational modifications and therefore would perhaps be more likely to be detected by anti HA antibody.
Chapter Two: Materials and Methods

2.1. Cells and Virus

2.1.1 Insect cell culture

_Spodoptera frugiperda_ 21 (Sf21) and _Trichopulsia ni._ (Hi-Five™) cells were maintained at 27°C in Graces Insect Medium (Invitrogen), and were supplemented with 10% foetal bovine serum (FBS) (Invitrogen) and 0.1% penicillin. Cells were routinely sub-cultured at 3 day intervals (when they reach about 80% confluency), in a 1:5 ratio. Sf21 cells were also grown in suspension, but for infection and assessment of virus activity, these cells were seeded as monolayers in T-25 or T-75 flasks. All aspects of cell culture maintenance were performed in a laminar flow hood.

2.1.2 Influenza virus strain, HA and NA

_cDNA clones of HPAI A/489/Hubei (H5N1) HA and NA in separate PMD-18T cloning vectors were obtained from Dr. Chongyi Zheng and sent by Dr. Qingzhen Liu, both from Wuhan University in China, and stored at -70°C. This strain of influenza virus was amongst one of the first isolated in Hong Kong during the initial HPAI H5N1 outbreak, and is considered to be of clinical significance by the WHO. Glycerol stocks of PMD-18T: NA and PMD-18T: HA were streaked on LB medium containing 50 µg/ml carbenicillin. The next day, individual colonies were picked, and overnight cultures were grown using 0.1% ampicillin to select for growth of bacteria carrying the gene of interest (either HA or NA)._

2.2 General DNA manipulation

2.2.1 Bacterial cultures and plasmid DNA isolation
Unless otherwise stated, all DNA clones were grown in DH5α *E. coli* cells on selective Luria-Bertani (LB) liquid or agar (16 mg/ml) growth media containing ampicillin (100 µg/ml) at 37°C. A single *E. coli* DH5α colony was picked, inoculated in 5 ml of LB supplemented with 0.1% ampicillin to select for growth of bacteria containing HA or NA plasmids, and rocked for 16 hours. Unless otherwise stated, plasmid DNA was isolated using either the GeneJET Plasmid Miniprep Kit, according to the manufacturer’s instructions (Fermentas Life Sciences or BioBasic). Unless otherwise stated, all DNA samples were subjected to electrophoresis at 15 volts/cm in 0.8% agarose gels supplemented with 200 ng/ml ethidium bromide.

2.2.2 Restriction enzyme digestions and polymerase chain reaction (PCR)

Unless otherwise stated, all restriction enzyme digestions were carried out in 50 µl reactions, using 1 µl of Fermentas Fast Digest restriction enzymes, 5 µl of 10x FastDigest Green buffer, and 5 µl of template, at 37°C for one hour. For cloning, PCR amplification was conducted using the Novagen *Kod* Hot Start Polymerase Kit and the BioRad MyCycler Thermal Cycler. In contrast, for PCR screening, *Taq* polymerase was employed. PCR conditions for both *Taq* and *Kod* polymerase, unless otherwise stated, are summarized in Table 2.1.

<table>
<thead>
<tr>
<th>Step</th>
<th>Kod Polymerase</th>
<th>Taq Polymerase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Polymerase</td>
<td>95°C for 5 Minutes</td>
<td>95°C for 5 Minutes</td>
</tr>
<tr>
<td>2. Denaturation</td>
<td>95°C for 20 seconds</td>
<td>95°C for 1 Minute</td>
</tr>
<tr>
<td>3. Annealing</td>
<td>Lowest Primer Tm for 10 seconds</td>
<td>Lowest Primer Tm for 30 seconds</td>
</tr>
<tr>
<td>4. Extension</td>
<td>70°C for 10 seconds /kb</td>
<td>70°C for 1 minute /kb</td>
</tr>
<tr>
<td>Repeat Steps 2-4</td>
<td>25 Cycles</td>
<td>25 Cycles</td>
</tr>
</tbody>
</table>
For cloning, PCR amplicons were purified using Wizard SV Gel and PCR Column Clean-up System (Promega) according to the manufacturer’s protocol.

2.2.3 Plasmid construction and cell transformation

DNA derived by PCR, restriction digestion, or plasmids were cloned into various vectors. Following electrophoresis, gel purification was accomplished using the Lamda Biotech Inc Direct-Gel Spin DNA Recovery Kit (Catalogue # D210). Ligation reactions were prepared using T4 DNA Ligase (Invitrogen) and 5x T4 DNA Ligase Buffer (Invitrogen), and incubated at room temperature for one hour or overnight at 4°C. Transformation of ligated DNA was conducted by incubating 5-7 µl of the ligation reaction with 100 µl of CaCl₂ competent E. coli DH5α cells on ice for thirty minutes, followed by a ninety second heat-shock at 42°C and incubated on ice for five minutes. Transformed cells were then plated on LB-containing agar plates supplemented with 50 µg/ml carbenicillin and incubated at 27°C overnight. The next day, individual colonies were picked and isolated as previously described.

2.3 Generation of recombinant virus constructs

2.3.1 Removal of the histidine tag (6x-His tag) from pFastBacB (Invitrogen)

To ensure effective detection and prevent potential complications with detection of recombinant HA, which has an N-terminal signal peptide that is cleaved during translocation to the ER, the 6x-His epitope tag was removed from pFastBacB via inverse PCR. Inverse forward and reverse primers were designed (Table 2.2) which amplified pFASTBacB, but excluded the start codon and the His-tag (Figure 2.1). Successful removal of the His-tag was confirmed by sequencing. The manipulated vector was named ΔHispFastBacB.
Figure 2.1 Schematic representation of ΔHispFastBacB. The Histidine tag has been removed via inverse PCR (Figure adapted from Invitrogen).
Table 2.2 Primers used for the generation of all recombinant baculoviruses. Restriction sites are italicized and underlined. Epitope tags are bolded and underlined.

<table>
<thead>
<tr>
<th>End Product</th>
<th>Gene</th>
<th>Forward 5’-3’</th>
<th>Reverse 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔHispFastBacB</td>
<td>FastBac</td>
<td>TTTGGGCCCCTGGATCC GGAATTCAAAAGGC</td>
<td>AAAAGGCCCTGGT TCGGACCGAGATCC</td>
</tr>
<tr>
<td>AcHA:FLAG</td>
<td>HA</td>
<td>AAACTCGAGAAAAATGG AGAAAATAATGTGCTTCTTC</td>
<td>AAAAGCTTACTTGT CGTCATCGTCTTTTGT AGTCATCTGAACCTAC AAATTAAATG</td>
</tr>
<tr>
<td>AcHA:GP64FLAG</td>
<td>GP64</td>
<td>AAAAGGATCCCTGCAGACTAG TGAGCAGTCGAACGCAGA AA</td>
<td>AAAAAAGCTTTTAATAT TGCTATTACGGTTTC</td>
</tr>
<tr>
<td>AcHA:GP64FLAG</td>
<td>HA</td>
<td>AAAAGGATCCCCAAAATGG AGAAAATAATGTGCTTCTTC</td>
<td>AAAACTAGTTTTGT CGTCATCGTCTTTTGT AGTCATCTGAACCTAC AAATTAAATG</td>
</tr>
<tr>
<td>AcVP39: HA</td>
<td>VP39</td>
<td>AAAAGGATCCATGCGGCCGCT GCAGGGAGGAGGAGGAAGTG CGCTAGTGCCCCGAGG</td>
<td>AAAAGCTTTTACCT GAGACTAGTGACGGC TATTCCTCCACCT</td>
</tr>
<tr>
<td>AcVP39: HA</td>
<td>HA</td>
<td>AAAACTAGTTGATGACTACAAA GACGATGACGACAAGCGAT TTGCACTTGGTACCATTG</td>
<td>TTTCCTCGAGGTAAG TTCTATTGATTTCA ATTTT</td>
</tr>
<tr>
<td>AcHA:VP39</td>
<td>VP39</td>
<td>AAAAGGATCCATGCGGCCGCGC TGCAGGGAGGAGAAGGAAGTG TGCGCTAGTGCCCCGAGG</td>
<td>AAAAGCTTTTACTC GAGACTAGTGACGGC TATTCCTCCACCT</td>
</tr>
<tr>
<td>AcHA:VP39</td>
<td>HA</td>
<td>AAAAGGATCTCTGGATGACTA CAAGACGATGACGACAAGC AGATTTGGCATTTACCATG</td>
<td>AAAAGCGCGCGCATGT AAAGTCCTATTGGATTC CAATTAT</td>
</tr>
<tr>
<td>AcNAHA</td>
<td>NA</td>
<td>AAAAGAGCTCATG AATCCAAATCAGAA GATAATAAC</td>
<td>AAAAGCTCTTACAAGC GTAATCTGGAACATC TATGCGACTTTGCTAC ATGGTGAATGGC</td>
</tr>
</tbody>
</table>
2.3.2 Generation of ΔHispFastBacBHA\textsubscript{FLAG}

After being prepared from an overnight culture, the HA ORF DNA was amplified via PCR to introduce 5’ XhoI and 3’ HindIII restriction sites (Table 2.2). Additionally, the sequence encoding a FLAG epitope tag (DYKDDDDK) was incorporated into the reverse primer to introduce the tag on the carboxyl end of HA (Table 2.2). Following PCR amplification and confirmation via gel electrophoresis (1% agarose, 15V/s), both HA and ΔHispFastbacB (Figure 2.2) were digested with XhoI and HindIII. The digested template and plasmid DNA were recovered, ligated and transformed into DH5α E.coli cells. Individual colonies were selected and PCR screens were conducted using primers specific to the multiple cloning site in the ΔHispFastbacB vector (Table 2.3) to ensure that the appropriate sized DNA band, of 2 kb was present when the PCR product was subjected to agarose gel electrophoresis. This construct was also confirmed to be correct by sequencing.

2.3.3. Generation of ΔHispFastBacBHA:VP39\textsubscript{FLAG} and ΔHispFastBacBVP39:HA\textsubscript{FLAG} (VP39 fusion constructs)

To generate the N-terminal fusion construct, ΔHispFastBacBHA:VP39\textsubscript{FLAG}, the wild-type VP39 ORF was PCR-amplified from AcΔCC, which represents the baculovirus bacmid with nothing cloned into the expression cassette. Primers used in this reaction also introduced 5’ BamHI, NotI and PstI restriction sites, respectively (Table 2.2), and 3’ SpeI, BamH1, and HindIII restriction sites, respectively. Both the PCR product and ΔHisFastbacB were digested with BamHI and HindIII at 37°C for 1 hour, and DNA was separated via agarose gel electrophoresis. Appropriate DNA fragments were extracted from the bands and purified from the gel, ligated and transformed into DH5α E. coli cells. The new construct, ΔHispFastBacBVP39 was sequenced.
Table 2.3 Primers used for PCR screening and sequencing of various clones in ΔHispFastbacB.

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>FastBac</td>
<td>TCTCGAGGCGATGCGGTACC</td>
<td>ACCGAGATCCGCGCCCG</td>
</tr>
<tr>
<td>Bacmid-Universal M13 Primers</td>
<td>GTTTTCCCAGTCACGAC</td>
<td>CAGGAAACAGCTATGAC</td>
</tr>
<tr>
<td>HA- Internal</td>
<td>GGAAAATGAGAGAACTCTAGAC</td>
<td>ATTCGTCACACATTGGGTTTCC</td>
</tr>
</tbody>
</table>
Next, to generate the HA:VP39\textsuperscript{FLAG} and VP39:HA\textsubscript{FLAG} fusions, HA was PCR-amplified, introducing 5′ BamHI and 3′ NotI, or 5′ SpeI and 3′ XhoI restriction sites, respectively. Additionally, in order to prevent problems with ER/Golgi trafficking of the VP39 fusions in the cell, both the 17 amino-acid N-terminal SP (MEKIVLLLALIVSLVKSD), and C-terminal TM domain (QILSIYSTVASSLALAIMVAGLSLWMCSN) as determined by Tatulian et al. (2000), were removed from HA in both PCR reactions. Further, the sequence encoding a FLAG epitope tag (N-DYKDDDDK-C) was incorporated into the reverse (for N-terminal fusion) or forward primer (for C-terminal fusion) to introduce the tag on either the N- or C-terminal end of HA. Both the PCR product and ΔHispFastBacB were digested with BamHI and NotI, for the HA:VP39\textsuperscript{FLAG} construct, or 5′ SpeI and 3′ XhoI for the VP39:HA\textsubscript{FLAG} construct. Digested vector and PCR product were then ligated and transformed. PCR screening of this vector using HA forward and FastbacB reverse primers was conducted (Table 2.3). Additionally, the cloned products were sequenced.

2.3.4 Generation of ΔHispFastBacBHA:GP64\textsuperscript{FLAG}

The HA ORF was PCR-amplified from the original PMD-I8T cloning vector in order to introduce a 5′ BamHI restriction site, a C-terminal FLAG tag and a 3′ SpeI restriction site (Table 2.2). Additionally, in order to ensure effective display and trafficking to the ER via the GP64 TM domain, the sequence coding for the 30- amino acid TM of HA (QILSIYSTVASSLALAIMVAGLSLWMCSN) as determined by Tatulian et al. (2000), was removed (Table 2.2). Both HA and ΔHisFastBacB were digested with BamHI and SpeI and cloned together as previously described. This construct, ΔHisFastBacBHA\textsubscript{FLAG} was sequenced.
To create ΔHispFastBacBHA:GP64\textsubscript{FLAG} fusion construct, the baculovirus ORF for major envelope protein GP64 was PCR-amplified from a previously constructed bacmid (Table 2.2), introducing a 5’BamHI and SpeI restriction site, as well as a 3’ HindIII restriction site. Both the PCR product and ΔHisFastBacBHA\textsubscript{FLAG} were digested with SpeI and HindIII for one hour at 37°C. Digestion reactions were subjected to agarose gel electrophoresis and appropriate bands were extracted and purified. HA was then ligated into ΔHisFastBacB, and after 1 hour incubation at room temperature, the reaction mixture was transformed into DH5α \textit{E.coli} and individual colonies were selected. Cloned plasmid DNA was sequenced.

2.3.5 Generation of ΔHisPFastbacBNA\textsubscript{HA}

The cDNA ORF of viral NA in a PMD-18T plasmid (constructed by Dr. Chongyi Zheng and sent by Dr. Qingzhen Liu, both from Wuhan University in China), was isolated from \textit{E. coli}. The NA ORF was PCR-amplified from PMD-18T using forward and reverse primers introducing a SacI restriction site to the 5’ end of NA and a HindIII restriction site on the 3’ end of NA (Table 2.2). Further, the reverse primer introduced a C-terminal HA-epitope tag to NA (N-YPYDVPDY-C). Both ΔHisPFastbacB and the NA\textsubscript{HA} PCR product were digested with HindIII and SacI, ligated and the resultant plasmid was transformed into \textit{E.coli}. The constructs were further purified as previously described and sequenced.

2.4 Construction, amplification and titration of recombinant baculoviruses

2.4.1 Generation of recombinant bacmids using the Bac-to-Bac System (Invitrogen)

Manipulated ΔHisFastBacB, now containing the gene of interest (GOI), was mixed with 50 µl of ΔCCBac+ Helper Cells (Bac-toBac, Invitrogen) and subjected to electric shock using a Gene Pulser (Bio-Rad) at 200 mV to initiate homologous recombination via the Tn7attn
transposition cassette. Transformants were then recovered at room temperature for four hours with shaking. Recovered, transformed cells were then diluted to $10^3$ in LB, and these dilutions were plated on LB containing-plates supplemented with 10 μg/ml kanamycin, 7 μg/ml gentamicin, 12 μg/ml tetracyclin, and 10 μg/ml chloramphenicol. EZ-Gal (Bioshop) was added to the plates to allow for blue/white colony selection. Plates were incubated overnight at 37°C. Transformed colonies were selected and amplified in overnight LB cultures supplemented with 7 μg/ml gentamicin, 50 μg/ml kanamycin, and 10 μg/ml chloramphenicol. Bacmid DNA was then purified from these cultures.

2.4.2 Purification of bacmid DNA

A modified version of the protocol that accompanies the EZ-10 Spin Column Plasmid DNA kit (Bio Basic) was used to purify bacmid DNA. Essentially, the protocol was employed until after the addition of the potassium acetate (Solution 3). After the 1-minute incubation, the samples were centrifuged for 5 minutes at 12,000 rpm. The supernatant was collected and mixed with 500 μl of isopropanol and incubated at room temperature for thirty minutes and the samples were centrifuged at 12,000 rpm for 10 minutes. The supernatant was discarded and 1 ml of 70% ethanol was added to each sample to wash the DNA. The samples were centrifuged again for 5 minutes at 12,000 rpm and the remainder of the ethanol was removed. Samples were then air-dried, and 20 μl of sterile elution buffer provided in the EZ-10 Spin Column Plasmid DNA kit (Bio Basic) was used to re-suspend the recombinant bacmid DNA. PCR screens using universal MI3 forward and reverse primers (Table 2.2) were conducted to ensure that an insert of the appropriate size was observed using gel electrophoresis. If no insert was present in the cloned bacmid, then a 3 kb band would be expected. If the bacmid contained the expected fragment however, a larger PCR product would be observed.
2.4.3 Transfection, amplification and titration of recombinant viruses

Approximately $9 \times 10^5$ Sf21 cells suspended in 2 ml of incomplete Grace’s Insect Medium were seeded in 35 mm dishes to obtain a final concentration of $4.5 \times 10^5$ cells /ml. Plates were incubated for one hour at room temperature to allow for attachment. For each transfection 5-10 µl of purified recombinant baculovirus DNA diluted in 100 µl of incomplete Grace’s Medium (ThermoScientific) was combined with 100 µl of Cellfectin (diluted 1:10 in incomplete Grace’s Medium). After a thirty minutes incubation at room temperature, 800 µl of incomplete Grace’s Medium was added to each Cellfectin-baculovirus DNA mixture to obtain a total volume of 1 ml, and this was then added to the plated cells. The cells were then incubated at 27°C for four hours. The transfection mixture was aspirated from the cells and replaced with 2 ml of complete Grace’s Medium, containing 5% FBS and 0.1% PenStrep (ThermoScientific).

After incubation at 27°C for forty-eight hours, both cells and medium were collected and the cells were pelleted by centrifugation (10 minutes, at 2,500 rpm). The supernatant was collected as the P1 recombinant virus stock, and cells were either discarded or reserved for future experiments. For amplification of recombinant viruses, approximately $2 \times 10^6$ Sf21 cells in 10 ml of complete Grace’s Insect Medium (ThermoScientific) were added to T-75 (Corning) tissue culture flasks. Ten to fifty µl of recombinant virus (the P1 stock) was then added to each flask, and the cells were incubated at room temperature for approximately one week, or until all cells had been successfully infected, which was determined via microscopy. At this point, cells and media were collected in 50 ml tubes and centrifuged at 2,500 rpm for five minutes to remove cellular debris. Supernatant was collected and stored at 4°C as the P2 recombinant virus stock.
All recombinant viruses were titrated using a serial end-point dilution method first described for baculoviruses by O’Reilly et al. (1992). Briefly, a 10 ml solution of Sf-21 cells and complete Grace’s Insect Medium at 1 x 10^5 cells/ml was prepared. Rows A-H and wells 1-10, and 12 of a 96-well microtitre plate were seeded with 100 µl of this suspension. A dilution series of recombinant baculovirus was prepared in 1.5 ml Eppendorf tubes. The 10^-1 dilution was prepared by adding 50 µl of rBV to 450 µl of complete Grace’s media. The 10^-2 dilution was prepared using 50 µl of the 10^-1 dilution, and 450 µl of complete Grace’s media. This procedure was repeated until a 10^-8 dilution of rBV was obtained. Nine-hundred µl of Grace’s media was added to each tube. BV dilutions were then aliquoted into a fresh disposable cell reservoir after which 30 µl of diluted BV was then dispensed into each of the first ten wells of Row A. This process was repeated with each dilution until wells 1-10 of rows A-H contained virus dilutions. Well 12 of each row was not inoculated with virus, to serve as an uninfected control. The plates were incubated for one week at 27°C. Each well was examined for cytopathic effect (CPE) at seven days post-infection (p.i.). Since CPE was difficult to identify in wells with a low virus concentration, a blind passage to fresh wells seeded with Sf21 monolayers was done at seven days p.i. and monitored for CPE for seven days. Titrations were calculated by the Reed and Muench method and expressed as a TCID_{50}/ml (Reed, 1983)

2.5 Infections and preparation of recombinant budded virions

2.5.1 Infection of cells with recombinant virus

For temporal analysis of protein expression in recombinant baculovirus infected Sf21 or Hi-Five insect cells, 1 x 10^6 cells were seeded per 35 mm dish, and were infected with the recombinant virus at a multiplicity of infection (MOI) of 10 (unless otherwise stated). At various
times p.i., infected cells were collected and pelleted by centrifugation (for 10 minutes at 3,000 x g)

2.5.2 One step growth curve

To assess BV production in each recombinant BV, the kinetics of viral growth were compared over a time course of 96-hours, at 12 hour intervals. Monolayers of Sf21 (3.5 x10^6) cells were seeded in 75 cm² flasks (Corning), and incubated at room temperature for 30 minutes to allow for cell attachment. Monolayers were then infected with one of each recombinant HA-expressing baculoviruses at an MOI of 1. Infections were carried out in duplicate. Infected cells were gently rocked for 1 hour at room temperature. Following virus infection, the virus dilution was replaced with fresh medium, and the cells were incubated at 27°C. At 0, 12, 24, 36, 48, 72 and 96 h.p.i., 1 ml aliquots were collected from the medium and centrifuged at 1,000 rpm for 5 minutes to pellet the cells. Supernatants (200 µl) were added to fresh microcentrifuge tubes, and stored at 4°C. For all virus infections, time “0” was considered to be the time immediately upon addition of virus. Viral titres were determined by end-point dilutions and titres were determined using the Reed-Muench method (Reed, 1983) (Appendix).

2.5.3 Recovery of infected cells and protein sample preparation

Cells infected with recombinant baculovirus were recovered via gentle scraping and centrifugation (5 minutes at 4,000 rpm) Supernatant was removed and discarded and the cell pellet was resuspended in, unless otherwise stated, 0.5% NP-40 (20 mM Tris, 100 mM NaCl, and 0.5% NP-40, pH 7.5). Occasionally, 1% radio-immuno precipitation (RIPA) (50 mM Tris, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS) lysis buffer was used. Additionally, cells were supplemented with the complete mini EDTA protease inhibitor (Roche).
In some instances, the whole cell lysates were further separated into soluble (nuclear and cytoplasmic) and insoluble (membrane) components. In these cases, the whole cell lysate was further centrifuged at 4°C at 12,000 rpm for five minutes. The supernatant was then aspirated and collected as the soluble fraction. The insoluble pellet was then re-suspended in 200 µl of lysis buffer (either NP40 or RIPA) and supplemented with 1 µl of benzonase (Fermentas) to increase the fluidity of the pellet and ease of loading. Crude cell lysates were stored at -70°C for later use.

2.5.4 Concentration of recombinant budded virions

At various times p.i. medium (containing recombinant budded virions) of Sf21 cells infected with recombinant baculoviruses, was collected. Medium was loaded onto a 3 ml sucrose cushion and subjected to ultracentrifugation at 126,000 rpm (using a SW32Ti rotor by Beckman Coulter) for 1 hour at 4°C. Supernatant was aspirated from the tube and the concentrated virion pellet was resuspended in 500 µl PBS (pH 6.2) and stored at 4°C.

2.5.5 Quantification of total protein (Bradford Assays)

For all Bradford Assays, the BioRad Protein Assay kit (Cat# 500-0001) was employed. Briefly, 1 part Dye Reagent Concentrate was diluted in 4 parts distilled H₂O. Ten µl of each of the ten BSA protein standards (concentrations ranging from 100 µg/ml to 1 mg/ml), along with protein samples diluted 1:10, were pipetted into microtiter plates (in triplicate). Two hundred µl of diluted Dye Reagent (diluted 1:5) was added to each well, and the plate was incubated at RT for five minutes. Absorbance was measured at 595 nm using a kinetic microplate reader (E2500, Tek-Trol Multiple Outlet, Molecular Devices), along with the computer program SOFTmax (version 2.35). The absorbance values for the BSA standards were used to generate a standard
curve. Using the equation of the linear trend line for the standard curve, the values of the unknown samples were extrapolated. Values for each sample were averaged to generate an average total protein concentration, and dilution factors were accounted for.

2.6 SDS-PAGE and Western immunoblot

2.6.1 SDS-PAGE

For all SDS-PAGE, 10 µl of 6x SDS-PAGE loading buffer supplemented with 2-mercaptoethanol was added to every 20 µl sample of cell lysate (both soluble and insoluble fractions) for a final SDS concentration of 2x. Unless otherwise stated, total cellular protein was measured for each Western blot and an equal quantity of protein was loaded into the gel. The samples were boiled at 100 °C for five minutes to denature proteins. Unless otherwise stated, 4 µl of PAGE-runner ladder was loaded onto the gel (BLUeye pre-stained protein ladder, FroggaBio). Two to ten ug of each sample was separated via SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Ten percent gels were prepared using recipes obtained from the Roche Lab FAQs Handbook, Section C, pages 152-153, and contained: dH2O, 10 % acryl-bisacrylamide (Bio-Rad), 4 M Tris for resolving [pH 8.8] and 5M Tris for stacking [pH 6.8] for resolving, 1% SDS, 1-5% ammonium persulfate and TEMED). The gels were then run at 150 V for 1 hour in running buffer (3% tris, 14.41 % glycine and 1% SDS).

2.6.2 Western immunoblotting

Following SDS-PAGE, samples were electroblotted onto polyvinylidene difluoride (PVDF) membranes at 100 V for 1 hour in 1 L of 1x transfer buffer (0.3% Glycine, 0.58 % Tris, 20% methanol, pH 7) in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) as directed by the manufacturer. Unless otherwise stated, all antibody solutions and membrane wash
solutions were composed of 1x Tris-buffered saline, supplemented with 0.1% Tween 20 (TBS-T) (50 mM Tris, 150 mM NaCl and 0.1% Tween 20) (pH 7.5). Proteins on PVDF membranes were incubated overnight with primary antibody at 4°C. Blots were then washed three times in 1xTBS-T for 10 minutes each, with shaking. The blots were then incubated in secondary antibody for 1 hour with gentle shaking. Blots were then washed 3 times for ten minutes in 1xTBS-T, and were then incubated in West Pico SuperBright Chemiluminescent Substrate (Pierce) for five minutes to detect specific proteins. X-ray film (Bioflex) was developed after varying exposure times.

Occasionally, and depending on the primary antibody, instead of chemiluminescence, a colorimetric reaction was conducted in which alkaline phosphatase conjugated NBT/BCIP substrate (ThermoScientific) was added directly to the membrane.

2.6.3 Coomassie blue staining to confirm equal loading

Coomassie blue staining was conducted to confirm equal loading of total protein quantitated via Bradford Assay. Briefly, 0.2 g of Coomassie Brilliant Blue (ThermoScientific) was dissolved in a solution of 20% methanol and 10% acetic acid, prepared in distilled H₂O. Following SDS-PAGE in which equal amounts of total protein were separated, 5 ml of the dye were added to the gel, and incubated overnight at room temperature with shaking. The following day the dye was removed, and the gel was de-stained in a 20% methanol, 10% acetic acid solution. Equal loading was visually assessed, and was considered to be successful when protein bands appeared to be equal in density and intensity across the blot.

2.7 Assessment of HA activity in insect cells

2.7.1 Collection and preparation of red blood cells
Chicken red blood cells (cRBCs) supplemented with Alsever’s Solution (Rockland Inc., Catalogue # R3020110) were gently washed three times with 5 ml PBS (pH 7.4) and centrifuged at 1,000 rpm for 10 minutes. The cell pellet was resuspended in PBS (pH 7.4) to a final concentration of either 1% or 0.5 %, for hemagglutination and hemadsorption assays, respectively.

2.7.2 Hemagglutination assays

The hemagglutination activities of HA derived from recombinant baculovirus-infected cells was determined at several times p.i. of Sf21. Cells were seeded in 35 mm dishes at a density of 1 x 10^6 and infected at an MOI of 10 with recombinant virus. At 48, 72 and 96 h.p.i., cells were aspirated from each dish and were centrifuged in an Eppendorf microfuge tube for 5 minutes at 1000 rpm to pellet detached cells. The supernatant fraction was aspirated from the tube, and retained as the budded virus fraction, and 1 ml of PBS was used to re-suspend the infected Sf21 cells. Both cell fractions and extracellular material were disrupted by sonication (3 x 30s intervals) (Mandel Scientific Model XL2020). One hundred µl of each sample (sonicates, whole infected cells and budded virus) was added to wells of a 96-well plate and two-fold serial dilutions were prepared. Fifty µl of 1% chicken RBCs were added to each well, which was incubated for 30 minutes at room temperature. The plates were examined for either the formation of a cross-linked matrix (indicating hemagglutination) or a button (indicating hemagglutination was absent).

In addition to assessing the hemagglutinating ability of infected cell lysates, the hemagglutination activity of whole (non-lysed) infected cells was also assessed. Cells were infected with one of each recombinant HA-expressing baculoviruses at an MOI of 10. At 48 h.p.i. infected cells were collected and counted using a hemocytometer and a phase contrast light
microscope. 3.5 x 10^5 cells from each infection (diluted in PBS) were added to the first well of a 96-well microtitre plate and diluted serially in a two-fold manner across the plate. After addition of the RBCs the plates were incubated for 30 minutes at room temperature and their hemagglutination reactions were evaluated. Newcastle disease virus (NDV) was the positive control and PBS was used as a negative control. Plates were read at 30 minutes.

The HA titre was the reciprocal of the last dilution where hemagglutination occurred.

2.7.3 Hemadsorption Assay

Approximately 1 x 10^6 Sf21 insect cells were seeded in 35 mm tissue culture dishes. Cells were infected at an MOI of 1 with various recombinant baculoviruses. At various times p.i., from 24 to 60 hours, medium was removed from the cells and the remaining cells were washed via gentle rocking three times with PBS (pH 6.2). Cells were then incubated with 4 ml of 0.5% chicken RBCs for 30 minutes, after which RBCs were removed, cells were washed several times in PBS (pH 6.2) and examined with a phase-contrast light microscope. The experiment was repeated several times, and at different MOIs of 1 and 10.

2.8 Examination of NA activity from insect cells

2.8.1 Neuraminidase assay

All work with NA was done with the assistance of James Ackford, an undergraduate project student working under my direction. The enzymatic activity of recombinant NA was assessed using the Amplex® Red Neuraminidase (Sialidase) Assay Kit (A22178, Invitrogen). Briefly, Sf21 cells were infected with AcNA_{HA} at an MOI of 10. At 96 h.p.i., cells were collected and lysed using NP40 lysis buffer supplemented with protease inhibitors (Complete Mini-EDTA, Roche). Fifty µl of the undiluted whole cell lysate was added to row A of a 96-well microtitre
plate and serially diluted in a two-fold manner. A positive control, NA derived from *Clostridium perfringens*, was diluted 50-fold in 1x reaction Buffer and 50 μl of this was added to the 96-well plate in triplicate. In addition, a 10 μM H₂O₂ positive product control was plated in triplicate along with a negative control containing only 1X reaction buffer. A 2X working solution was made using 50 μl of 10 mM Amplex Red reagent stock solution, 10 μl of 100 U/ml horseradish peroxidase stock solution, 100 μl of 200 U/ml galactose oxidase stock solution, 250 μl of 10 mg/ml fetuin stock solution, and 4.59 ml of 1X Reaction Buffer. Fifty μl of the 2X working solution was added to all samples and controls using a multi-channel pipette. The 96-well plate was incubated for 30 minutes at 37 °C, shielded from light. Absorbance was measured at 560 nm, and the empty wells were used as a blank for absorbance readings and these values were subtracted from all samples and controls. Each sample was assessed in triplicate.
Figure 2.2 Schematic representation of the recombinant baculoviruses generated. All GOIs were first cloned into ΔHispFastBacB, and then subsequently electroporated into the mini Tn7 _attm_ transposition sites present in the AcΔCC recombinant bacmid, under the very late polyhedrin promoter. AcΔCC contains the entire baculovirus genome, but lacks the chitinase/cathepsin genes.
Chapter 3: Results

3.1 Generation of recombinant viruses

The overall goal of this research was to assess and compare the effectiveness of three baculovirus expression methods, specifically protein overexpression, surface display and capsid display for production of influenza virus HA. Recombinant viruses were generated using the commercially available Bac-to-Bac BEVS system (Invitrogen).

3.1.1 Development of the baculovirus recombinant HA constructs

Four recombinant HA-expressing viruses were generated: AcHA_{FLAG}, AcHA:GP64_{FLAG}, AcHA:VP39_{FLAG} and AcVP39:HA_{FLAG} through the corresponding bacmid intermediates (Fig. 3.1). AcHA_{FLAG} represented the traditional overexpression system and included the entire HA ORF with its signal peptide. For AcHA:GP64_{FLAG}, the HA_{FLAG} was inserted at the amino end of GP64, using the signal peptide of GP64. For this, the HA:GP64_{FLAG} should be processed and located in the plasma membrane of the cell and envelope of the virus. The normal GP64 was retained to ensure proper virus budding. For AcHA:VP39_{FLAG} and AcVP39:HA_{FLAG} the HA signal peptide and transmembrane domains were removed to ensure HA did not go to the ER or plasma membrane, respectively. Additionally, normal VP39 was retained in the virions to ensure proper nucleocapsid formation. In all cases, the different constructs were driven by the polyhedrin promoter. To confirm proper cloning, each bacmid was PCR-screened using M13 specific primers (Table 2.3), and the PCR products were separated by agarose gel electrophoresis. All recombinant bacmids contained DNA fragments of the expected sizes. Sequence analysis confirmed that all recombinant-HA expressing bacmids were correct.
Figure 3.1: Schematic representation of the HA-expressing constructs in ΔHispFastbac B. The HA was cloned into the bacmid polyhedrin locus and under the polyhedrin promoter (Polh P). Bacmids were verified via PCR, using M13 forward and reverse primers. Additional sequencing with forward and reverse primers specific to HA verified that the fusion constructs were correct. All DNA fragments were cloned into TN7R and TN7L transposition sites which allow for homologous recombination in E. coli. The signal peptide (SP) of HA was manipulated for various constructs, as was the C-terminal transmembrane domain (TM). A FLAG-epitope tag was fused to either the C or N terminus of HA to allow for simple detection of all recombinant expressed HA proteins. SV40 is a polyadenylation signal that is not recognized by the polyhedrin promoter. Gm’ represents the gentamycin resistance gene which allows for selection. GP64 is the baculovirus major envelope glycoprotein, and VP39 is the baculovirus major capsid protein.
3.1.2 Viral growth kinetics

Viral growth kinetics were compared among the four recombinant HA-expressing viruses and the reference AcΔCC to determine whether the alterations to the baculovirus genome affected virus replication and growth (Fig 3.2). AcΔCC was the backbone for the constructs and lacks the chitinase and cathepsin genes to protect the HA from proteolytic cleavage. Approximately 3x10⁶ Sf21 cells were seeded in T-75 flasks and infected at an MOI of 0.1 with each of the four recombinant viruses, along with AcΔCC. All infections were carried out in duplicate. Fifty µl of medium containing the budded virions from each infection was collected at twelve-hour intervals from 12 to 96 h.p.i. Infectious budded virus in the extracellular medium was titred using the TCID₅₀ titration method (Reed, 1983). The growth rate of infectious budded virions was comparable among the viruses. Statistical analysis using two-factor ANOVA confirmed that virus titres at each time point were not statistically different from one another, with 95% confidence (p-value > 0.05) (see appendix for Microsoft Excel output).

3.2 Expression of HA by three different methods as measured using anti FLAG antibody

Expression of HA by the four different recombinant baculoviruses was followed by Western immunoblots of infected cells using an anti-FLAG antibody to determine the size and relative level of the expressed HA.
Figure 3.2 Comparison of recombinant virus replication in Sf21 cells. Mean budded virus titres in the supernatants of Sf21 cell infected at an MOI of 1 were determined by TCID\textsubscript{50}. AcΔCC and all four recombinant HA-expressing viruses had similar growth kinetics.
3.2.1 HA\textsubscript{FLAG} overexpression

To determine whether transfected Sf21 cells expressed recombinant HA\textsubscript{FLAG}, cells transfected with each of two clones of BAC.HA\textsubscript{FLAG} were collected at 48 h.p.t., resuspended in PBS, pelleted and lysed. Proteins were separated by SDS-PAGE, blotted and probed using a monoclonal anti-FLAG antibody (Sigma). AcCHIA\textsubscript{FLAG}, a recombinant virus expressing a FLAG-tagged baculovirus chitinase protein was the positive control for the anti-FLAG antibody. A band at 58 kDa (Fig. 3.3, lane 3) was detected corresponding with that of CHIA\textsubscript{FLAG}, indicating the ability of the antibody to detect FLAG-tagged protein. Lysate from Sf21 cells infected with AcΔCC (empty bacmid, lane 4) was the negative control for the anti-FLAG antibody, and as expected, no band was detected for AcΔCC. The anti-FLAG antibody identified two specific bands of BAC.HA\textsubscript{FLAG} 1 and 2 (lanes 1 and 2, respectively). These bands migrated at 74 kDa and 25 kDa, respectively, as determined using linear regression of marker band migration (see appendix for sample calculation). In lane 1, a very faint 65 kDa band was also visible. This band was not observed for the BAC.HA\textsubscript{FLAG} cell lysate in lane 2, which appeared to have less protein than lane 1. BAC.HA\textsubscript{FLAG} from lane 1 was selected for virus amplification and further experiments.

3.2.2 AcHA\textsubscript{FLAG} expression in S21 cells

Temporal expression of HA\textsubscript{FLAG} was examined in Sf21 cells by Western immunoblot. Sf21 cells were infected at an MOI of 10 with AcHA\textsubscript{FLAG}, and collected at intervals from 24 to 96 h.p.i. Ten µg of protein from each lysate was separated by SDS-PAGE, blotted, and the blot was probed using the anti-FLAG antibody (Fig 3.4A).
Figure 3.3 Western immunoblot (12% PAGE) of lysate from Sf21 cells transfected with Bac.HA\textsubscript{FLAG} at 48 hours h.p.t. MW is molecular weight marker. Calculated protein sizes are represented on the right side of the figure. Lanes 1 and 2: Sf21 cells transfected with BacHA\textsubscript{FLAG}1 and BacHA\textsubscript{FLAG}2. Lane 3: Sf21 cells infected with AcCHIA\textsubscript{FLAG}. Lane 4: Sf21 cells infected with bacmid containing only the empty expression cassette (AcΔCC). The blot was probed with a primary murine anti-FLAG antibody (diluted 1:20,000 in TBS-T) and a secondary anti-mouse antibody (diluted 1:10,000 in TBS-T).
Figure 3.4 Western immunoblot of AcHA\textsubscript{FLAG} in Sf21 (A) and Hi-Five\textsuperscript{TM} (B) infected cell lysate from 24 to 96 h.p.i. MW is molecular weight marker. Calculated protein sizes are represented on the right side of the figure. For Sf21 cells, 10 µg of lysate was used while for Hi-Five\textsuperscript{TM} cells, only 5 µg of lysate was used. Primary murine anti-FLAG antibody diluted 1:20,000. The secondary antibody was an anti-mouse HRP-conjugated antibody (1:10,000). Controls included lysates from Hi-Five\textsuperscript{TM} cells infected with either AcCHI\textsubscript{FLAG} (FLAG control), or AcΔCC (negative) at 96 h.p.i. Lysates from mock infected Hi-Five\textsuperscript{TM} cells were also probed with anti-FLAG Ab as an additional negative control. (C) Schematic of HA\textsubscript{FLAG} construct.
Three bands were detected in the lysate from Sf21 cells infected with AcHA\textsubscript{FLAG} and collected from 48 to 96 h.p.i. (Fig 3.4A), but no bands were detected at 24 h.p.i. The three bands migrated at 74 kDa, 65 kDa and 25 kDa, respectively. Both the 74 and 65 kDa bands were prominent in each lane. The intensity of each band increased from 48 to 96 h.p.i. The expected 58 kDa band was detected for CHIA\textsubscript{FLAG}. No bands were detected in Sf21 cells infected with either AcΔCC and mock infected Sf21 cells at 96 h.p.i.

3.2.3 AcHA\textsubscript{FLAG} expression in Hi-Five\textsuperscript{TM} cells

AcHA\textsubscript{FLAG} expression was also monitored in Hi-Five\textsuperscript{TM} cells to compare with the expression in Sf21 cells. Hi-Five\textsuperscript{TM} cells were infected at an MOI of 10 and collected from 24 to 96 h.p.t. Five µg of total protein from each lysate was separated via SDS-PAGE prior to Western blotting (Fig 3.4B).

HA\textsubscript{FLAG} expression in Hi-Five\textsuperscript{TM} cells was similar to that in Sf21 cells. While no bands were seen at 24 h.p.i., there were two bands at 74 kDa and 65 kDa respectively from 48 to 96 h.p.i. Compared to Sf21 cells very little of the 25 kDa protein was detected in Hi-Five\textsuperscript{TM} cells, which was detected only faintly at 96 h.p.i. (Fig 3.4B). The 65 kDa band in Hi-Five\textsuperscript{TM} cells was more intense than the 74 kDa one. This result was opposite to what was observed in Sf21 cells, in which the 74 kDa band was stronger. The 58 kDa CHIA\textsubscript{FLAG} band was detected in lysate from Hi-Five\textsuperscript{TM} cells infected with AcCHIA\textsubscript{FLAG}. No bands were detected in lysates from Hi-Five\textsuperscript{TM} cells infected with AcΔCC or in mock-infected Hi-Five\textsuperscript{TM} cells.
3.2.4 Comparison of HA<sub>FLAG</sub> recovery between NP40 and RIPA lysis buffers for ELISA

The ultimate goal of the recombinant HA is for it to be used as antigen in ELISA. Since ELISA plates are generally coated with soluble antigen, an experiment was conducted to compare the recovery of HA<sub>FLAG</sub> in the soluble fraction of infected Hi-Five<sup>TM</sup> cells lysed with either NP40 or RIPA lysis buffers from 48 to 96 h.p.i. Hi-Five<sup>TM</sup> cells were grown in serum-free medium and were infected with AcHA<sub>FLAG</sub> at an MOI of 10. Cells were then lysed with either NP40 buffer or RIPA buffer (lanes N and R, respectively in Fig 3.5), and total proteins were separated via SDS-PAGE. The expected 65 kDa protein that corresponds with full-length HA<sub>FLAG</sub> was easily detectable with the anti-FLAG antibody (Fig 3.5). At 96 h.p.i. a second band was distinguishable at 74 kDa for both buffers. A band at this size was also observed in previous infections of Sf21 and Hi-Five<sup>TM</sup> cells, but at earlier times p.i. In general the intensity of the bands was similar regardless of the lysis buffer employed. However, the bands resulting from cells lysed with RIPA appear slightly more intense at 72 and 96 h.p.i. compared to NP40. The upper 74 kDa band was observed in Figures 3.3 and 3.4 but was much less distinct (Fig 3.5) when only the soluble fraction was used in the Western blot, and in Hi-Five<sup>TM</sup> cells grown in the absence of FBS. It is possible that the 74 kDa band corresponds to the full-length HA<sub>FLAG</sub> fusion, and the 65 kDa band corresponds to an HA cleavage product. Further investigation was required to determine the identity of the 65 and 74 kDa protein bands in Sf21 and Hi-Five<sup>TM</sup> cells infected with AcHA<sub>FLAG</sub>. 

58
Figure 3.5 (A) Western immunoblot of temporal expression of HA_{FLAG} in Hi-Five^{TM} cells. Protein was detected using anti-FLAG antibody (1:20,000), and a secondary anti-mouse antibody (1:10,000). MW is molecular weight marker. The times p.i. are indicated above the lanes, and the lysis buffer employed, either NP40 (N) or RIPA (R), are indicated below the lanes. Calculated protein sizes are represented on the right side of the figure. The positive FLAG control was CHIA_{FLAG} and the negative control was AcΔCC. (B) Schematic representation of the recombinant HA_{FLAG} construct.
3.2.5 Nature of 65 and 74 kDa bands: serum–containing versus serum-free media

Western blots of cell lysates of cells grown in the presence of FBS showed strong expression of both 65 and 74 kDa bands (Fig 3.4), while cells grown in serum-free medium had a less intense 74 kDa band, detectable only at 96 h.p.i. (Fig 3.5.) To determine if this was reproducible, AcHAFLAG expression was followed in Hi-Five™ cells grown in either serum-free or FBS-supplemented medium for the cleavage and the relative amounts of the two proteins were monitored.

Cells were infected at an MOI of 10. At 72 h.p.i. infected cells were collected, lysed and proteins were separated using SDS-PAGE and subjected to Western immunoblot. There was no qualitative difference in HAFLAG expression in Hi-Five™ cells grown in either serum-free medium or serum-containing medium (Fig 3.6). Both the 74 and 65 kDa forms of HAFLAG were detected, regardless of the type of medium used, and therefore it was concluded that presence or absence of serum was not responsible for the differential detection of the 65 and 74 kDa protein bands. However, it was noted that there was less of the 74 kDa band in serum-free medium as also seen in figure 3.5, corroborating these earlier results.

3.2.6 HAFLAG expression in Sf21 cells in the presence or absence of tunicamycin

Since presence or absence of serum did not alter the detection of the 65 and 74 kDa bands, it was rationalized that perhaps the 74 kDa band for AcHAFLAG was a result of glycosylation of HA. To investigate this possibility, Sf21 cells were infected with AcHAFLAG, and supplemented with tunicamycin (10 µg/ml), a known inhibitor of glycosylation. Infected cells were collected at 48 h.p.i. For AcHAFLAG infected Sf21 cells treated with tunicamycin, only the 65 kDa band was detected (Fig 3.7, lane 2). However both the 65 and 74 kDa bands
Figure 3.6 Western immunoblot of HA\textsubscript{FLAG} expression in Sf21 cells at 72 h.p.i., grown in the presence (+FBS) or absence (-FBS) of serum. MW is molecular weight marker. Protein bands at 74 and 65 kDa were detected using a primary murine anti-FLAG antibody (1:20,000), and a secondary anti-mouse antibody (1:10,000). The FLAG control was CHI\textsubscript{FLAG} and the negative controls were Ac\Delta\textsubscript{CC} infected Sf21 cells and mock-infected Sf21 in the presence of serum.
Figure 3.7 Western immunoblot showing that in the presence of tunicamycin at 48 h.p.i. (lane 2), the 74 kDa band normally observed in untreated. MW is molecular weight marker. Sf21 cells infected with AcHA_{FLAG} (lane 1), is not detectable when probed with anti-FLAG antibody (1:20,000). The virus or treatment used is indicated above each lane. Below the lanes + and – refer to the presence or absence of tunicamycin (TUN) or DMSO (DMSO), respectively. Calculated protein sizes are represented on the right side of the figure.
were detected in Sf21 cells in the absence of tunicamycin (Fig 7, lane 1). Cells were also treated with DMSO, the carrier for tunicamycin, to ensure that it was not responsible for the absence of the 74 kDa band. In the presence of DMSO only, there was a strong 74 kDa band but only a very faint 65 kDa band (Fig 3.7, lane 3). It was expected that both bands would be detected at levels similar to each other and similar to that in lane 1. Nonetheless, because the 74 kDa band remained intense in the presence of DMSO, it is unlikely that DMSO is responsible for the observed effect of tunicamycin on cells infected with AcHAFLAG. These results suggested that the 74 kDa protein strongly expressed in both Sf21 and Hi-Five™ cells infected with AcHAFLAG was a consequence of glycosylation, and that the 65 kDa band represents HA0, the full-length, uncleaved and non-glycosylated protein.

3.2.7 AcHA:GP64FLAG expression

The second construct, AcHA:GP64FLAG, was designed to have the HA fused to the amino end of the major envelope glycoprotein GP64 using the HA signal peptide. The genome still retained the native GP64 gene to ensure that virus budding was not compromised by fusion of the HA to GP64.

HA:GP64FLAG expression was monitored in Sf21 cells infected with AcHA:GP64FLAG at an MOI of 10 and collected at 72 h.p.i. Two µg of total protein was separated via SDS-PAGE and analyzed by Western immunoblot using the anti-FLAG antibody (Fig 3.8A).

When probed with anti-FLAG Ab, HA:GP64FLAG was detected at 72 h.p.i. as a band at 130 kDa (lanes 1), which corresponds in size with the full-length HA protein fused to both the FLAG epitope tag and the GP64 major fusion protein (Figure 3.8C). Additionally, there was a faster migrating band at 90 kDa, which corresponded to the cleaved HA2 subunit (27 kDa),
Figure 3.8 Western immunoblot of (A) Sf21 cells or (B) Hi-Five™ cells infected with AcHA:GP64\textsubscript{FLAG} at 72 h.p.i. MW is molecular weight marker. Two µg of each sample was loaded in duplicate, and probed with either the anti-FLAG antibody (1:20,000) (left panels), or a monoclonal murine anti-GP64 antibody, (1:10,000) (right panels). The secondary antibody was anti-mouse HRP conjugated antibody (1:10,000). Calculated protein sizes are represented on the right side of each blot. Lane 1 (A) and (B): cells infected with AcHA:GP64\textsubscript{FLAG}. Lane 2 (A): Sf21 cells infected with AcCHIA(ASD)\textsubscript{FLAG} Lane 2 (B): Hi-Five™ cells infected with AcCHIA\textsubscript{FLAG} Lane 3 (A) and (B): AcΔCC infected cells Lane 4 (A) and (B): mock-infected cells (C). Schematic representation of the fusion construct in which the HA TM domain was removed, and HA was fused with FLAG at its carboxy terminus. HA\textsubscript{FLAG} was then fused to the N terminus of GP64. The N-terminal HA SP was retained.
fused with the FLAG epitope tag, followed by the GP64 protein (64 kDa) (Fig 3.8 B). The expected band at 45 kDa was detected for CHIA(ASD)_FLAG, a different FLAG control expressing only the active site domain (ASD) of chitinase rather than the full-length protein (lanes 2). No bands were detected for Sf21 cells infected with AcΔCC and mock infected Sf21 cells (lanes 3 and 4, respectively).

Alternatively when Sf21 cells infected with AcHA:GP64_FLAG were probed with monoclonal anti-GP64 antibody, three bands at 130 kDa, 90 kDa and 64 kDa, respectively, were detected. The 130 and 90 kDa bands corresponded to those detected by the anti-FLAG antibody. The lower band at 64 kDa corresponded to the wild-type GP64, which was purposefully retained in the recombinant baculovirus, in addition to the HA:GP64 fused version. A single 64 kDa protein band corresponding to the wild-type GP64 protein was detected for AcΔCC (lane 3). This band was very faint for CHIA(ASD)_FLAG (lane 2), perhaps because only the soluble fraction of cells infected with AcCHIA(ASD)_FLAG was blotted, and GP64, as a membrane protein, would be expected to remain mostly insoluble whereas total cell lysate was blotted for AcΔCC and AcHA_FLAG samples. No bands were detected for the mock infected Sf21 cells (lane 4).

The same experiment was repeated in Hi-Five™ cells, with the exception of the positive FLAG control, which was total protein from cells infected with AcCHIA_FLAG. When probed with anti-GP64 Ab, the expected 64 kDa band for CHIA_FLAG was also very faint. In this case it was perhaps because the titre of this virus was about 100 fold less than that of all other viruses used in these experiments. Otherwise, this experiment confirmed qualitatively that AcHA:GP64_FLAG expression between Sf21 and Hi-Five™ cells was comparable.

3.2.8 AcHA:VP39_FLAG and AcVP39:HA_FLAG expression
The recombinant baculoviruses AcHA:VP39\textsubscript{FLAG} and AcVP39:HA\textsubscript{FLAG} were designed to have HA fused to the major capsid protein VP39 at its amino and carboxy termini, respectively. The genome for each recombinant virus still retained the wild-type VP39 gene to ensure that nucleocapsid formation was not compromised by fusion of HA to VP39.

Sf21 cells were infected with either AcHA:VP39\textsubscript{FLAG} or AcVP39:HA\textsubscript{FLAG} and monitored for HA:VP39\textsubscript{FLAG} and VP39:HA\textsubscript{FLAG} expression at 72 h.p.i. by probing with the anti-FLAG antibody and a monoclonal anti-VP39 antibody (Fig 3.9A). A 104 kDa protein band was detected for AcHA:VP39\textsubscript{FLAG} and AcVP39:HA\textsubscript{FLAG} infected Sf21 cells using the anti-FLAG antibody (Fig 3.9A, lanes 1 and 2, left panels). This size reflects the size of the 39 kDa VP39 protein fused with the 65 kDa HA\textsubscript{FLAG} or FLAGHA. A band of the expected size of 58 kDa was detected for CHIA\textsubscript{FLAG} (lanes 3) while no bands were detected for Sf21 cells infected with AcΔCC or mock-infected cells (lanes 4 and 5, respectively).

A similar band at 104 kDa which was detected for HA:VP39\textsubscript{FLAG} and VP39:HA\textsubscript{FLAG} when probed with anti-FLAG Ab (lanes 1 and 2) was observed using anti-VP39 (lanes 5 and 6) antibody, and reflects the size of the full-length HA:VP39 fusion protein (Figure 3.9B). In addition to the 104 kDa band, a 39 kDa band was detected with the anti-VP39 antibody (lanes 6-9). This band corresponded to wild-type VP39. CHIA\textsubscript{FLAG} and AcΔCC (lanes 8 and 9 respectively) also yielded a 39 kDa band, representing wild-type VP39, which is present in all baculoviruses. The 39 kDa band for CHIA\textsubscript{FLAG} was very faint (lane 8), likely due to the low titre of the AcCHIA\textsubscript{FLAG}. 
Figure 3.9 Western immunoblots of (A) Sf21 or (B) Hi-Five™ cells infected with either AcHA:VP39\textsubscript{FLAG} or AcVP39:HA\textsubscript{FLAG}. (lanes 1 and 2 and 6 and 7, respectively, on each blot) at 72 h.p.i. MW is molecular weight marker. For (A), the FLAG control was AcCHIA\textsubscript{(ASD)FLAG}, in lanes 3 and 8. For (B), the FLAG control was AcCHIA\textsubscript{FLAG}, in lanes 3 and 8. Lanes 4 and 5 and 9 and 10 for both (A) and (B) contained lysates for AcΔCC and mock infected cells, respectively. Two µg of each sample was loaded in duplicate and the blots were probed with either anti-FLAG antibody on the left panel of A and B (1:20,000) or a monoclonal murine anti-VP39 antibody on the right panel of A and B (1:10,000). All samples were probed with a secondary anti-mouse HRP conjugated antibody (1:10,000). Calculated protein sizes are represented on the right side of each blot. (C) Schematic representation of the fusion construct in which both the HA TM domain and signal peptides were removed, and HA was fused with FLAG at either its carboxy (N-terminal fusion) or amino (C-terminal fusion) terminus. HA\textsubscript{FLAG}/\textsubscript{FLAG}HA was then fused to the N or C terminus of VP39.
virus. Finally no bands were detected for mock infected Sf21 cells lysate using the anti-VP39 antibody.

The same experiment was repeated with Hi-Five™ cells, and the protein expression for the two cell lines was similar (Fig 3.9B).

### 3.2.9 Relative levels of HA expression among all HA-expressing baculoviruses

The main objective of this research was to determine which baculovirus expression method is the most efficient and economical for production of HA as an antigen. HA expression levels among all recombinant HA-expressing baculoviruses were compared using anti-FLAG Ab. Sf21 cells infected at an MOI of 10 with each of the four recombinant baculoviruses expressing HA were collected at 72 h.p.i. Two µg of each lysate was separated by electrophoresis and the proteins were transferred and probed with the anti-FLAG antibody. The Western blot (Fig 3.10) shows that when the same amount of protein was loaded, HA:GP64\textsubscript{FLAG} showed the most intense bands (at 130 and 90 kDa), followed by HA\textsubscript{FLAG}, (at 74 and 65 kDa) and then by VP39:HA\textsubscript{FLAG} (at 104 kDa). Intensity of the bands for HA:VP39\textsubscript{FLAG} (104 kDa) was the weakest in which HA was cloned into the N-terminus of VP39. No bands were detected for AcΔCC and mock-infected Sf21 cell lysates.

### 3.2.10 Detection of HA in budded virions from HA recombinant virus infected cells

To determine whether FLAG-tagged HA was present in the budded virions, Sf21 cells were infected with each of the recombinant viruses at an MOI of 1. At 96 h.p.i., cells were pelleted and discarded, while supernatant containing budded virions was collected, and concentrated by ultracentrifugation. Budded virions were lysed in NP40 lysis buffer, and 5 µg of
Figure 3.10 Comparison of HA expression by recombinant HA-expressing baculoviruses, along with AcΔCC and mock infected Sf21 cells at 72 h.p.i. Cells were infected at an MOI of 10. MW is molecular weight marker. Two µg of each cell lysate sample was loaded and probed with the anti-FLAG antibody (1:20,000) followed by a secondary anti-mouse HRP conjugated antibody (1:10,000). Calculated protein sizes are represented on the right side of the figure. All bands were at the expected sizes, as previously described.
Figure 3.11 Western immunoblot of concentrated budded virions of the recombinant HA-expressing viruses from infections of Sf21 cells using an anti-FLAG (left panel) or anti-GP64 antibody (right panel). Molecular weight is represented on the left side of the figure. AcΔCC was used as a negative control.
each lysate was subjected to Western immunoblot and probed with anti-FLAG antibody. FLAG-tagged HA was detectable in the budded virions of all HA-expressing baculoviruses, but not in budded virions of AcΔCC (Fig 3.11). However, the bands detected by anti-FLAG Ab in the budded virions of AcHA:GP64\textsubscript{FLAG} and AcHA\textsubscript{FLAG} (130 kDa and 74 and 65 kDa, respectively) were much stronger than those of the two VP39 display viruses, both of which showed only faint bands of the expected size of 104 kDa, and only when the blot was over-exposed. Further, budded virions of AcHA\textsubscript{FLAG} contained the glycosylated 74 kDa protein. This suggests that post-translational modifications occurred efficiently during the processing of HA.

The GP64 antibody (Fig 3.11 right panel) was the positive control for the budded virions. Bands for VP39:HA\textsubscript{FLAG}, HA:VP39\textsubscript{FLAG}, HA:GP64\textsubscript{FLAG}, and HA\textsubscript{FLAG} and AcΔCC were detected at the expected size of 64 kDa, confirming that the budded virions were present. In addition to the 64 kDa band corresponding to full-length GP64, two bands at 90 and 130 kDa were detected for HA:GP64\textsubscript{FLAG}, but they were very faint on the blot, and were most apparent when the blot was over-exposed.

3.3 Recognition of HA expressed by recombinant viruses by antibodies against HA

Previously, expression of HA by the four recombinant viruses was demonstrated by Western blot with an anti-FLAG antibody. However, for the HA to be useful for diagnostic purposes they also have to be able to react with anti-HA antibodies. For this, the HA expression in cells was monitored using a variety of antibodies against HA to provide more conclusive evidence for immune recognition of HA.

3.3.1 Detection of HA\textsubscript{FLAG} with a commercial polyclonal, mono-specific (H5) HA antibody
To determine whether HA\textsubscript{FLAG} can be detected by a commercially available rabbit polyclonal anti-H5 HA antibody (ProSci, Catalogue #3425) Sf21 cells were infected with AcHA\textsubscript{FLAG} at an MOI of 10, collected at 72 h.p.i. and lysed. Bands suggestive of both the glycosylated (74 kDa) and non-glycosylated (65 kDa) forms of HA\textsubscript{FLAG} were detected from 48 to 96 h.p.i. (Fig 3.12, lanes1-4). HA expression by a fowl-adenovirus vector (FAdV) in which the HA was cloned in the rightward (FAdV-HA\textsubscript{R}) or leftward (FAdV-HA\textsubscript{L}) orientations was also followed. Chicken liver cells (CH-SAHS) infected with FAdV-HA\textsubscript{R} and FAdV-HA\textsubscript{L} recombinant viruses yielded a strong band at 55 kDa, detected by the anti H5 HA antibody and a faint one at 74 kDa. No HA protein was detected for the empty FAdV vector, FAdV9Δ4 infected CH-SAHS cells (lane 8). For AcCHIA\textsubscript{FLAG} or AcΔCC, neither the 74 kDa nor the 65 kDa bands were detected (lanes 7 and 9, respectively). No bands were detected for mock-infected avian CH-SAHS or insect Sf21 cells (lanes 10 and 11, respectively). There was an unexpected, specific 45 kDa band detected in cells infected with AcHA\textsubscript{FLAG}, AcCHIA\textsubscript{FLAG} and AcΔCC, but not in mock-infected Sf21 cells. Because this 45 kDa band was not detected in Sf21 cells, this protein, detected by the polyclonal anti-H5 antibody must be present in cells infected with any baculovirus.

3.3.2 Detection of HA from recombinant HA-display and HA\textsubscript{FLAG} viruses using a commercial monospecific polyclonal, anti-HA antibody

To determine whether Sf21 cells infected with each HA-expressing display virus reacted with the commercial polyclonal anti-H5 HA antibody #3425, Sf21 cells were infected with each of the recombinant baculoviruses at an MOI of 10, collected at 72 h.p.i. and lysed. When probed with the anti-H5 antibody, lysates from both C and N terminal VP39 display viruses yielded bands at the expected size, at 104 kDa (Fig 3.13 lanes 1 and 2, respectively). An expected band
Figure 3.12 Western immunoblot showing lysates of Sf21 cells infected with AcHA<sub>FLAG</sub> and probed with a polyclonal rabbit anti-HA antibody (ProSci, diluted 1:10,000), from 24 to 96 h.p.i. (lanes 1-4). Lanes 5 and 6 are FAdV-HA (R and L, respectively) infected CH-SA cells, showing a doublet. Controls were cells infected with AcCHIA<sub>FLAG</sub> (Sf21, lane 7), FadV-<del>9Δ4</del> (CH-SA, lane 8), AcΔCC (Sf21, lane 9), as well as mock infected CH-SA (lane 10) and Sf21 cells (lane 11). Molecular weight marker is represented on left. Calculated protein sizes are represented on the right side of the figure.
Figure 3.13 Western immunoblot of Sf21 cells infected with AcVP39:HA_{\text{FLAG}} and AcHA:VP39_{\text{FLAG}} (lane 1 and 2, respectively), AcHA:GP64_{\text{FLAG}} (lane 3) or AcHA_{\text{FLAG}} (lane 4). Lanes 5 and 6 are FAdV-HA (R and L, respectively) infected CH-SAH cells. Lanes 7-11 include cells infected with AcCHIA_{\text{FLAG}} (Sf21, lane 7), AcΔCC (Sf21, lane 8), and mock infected Sf21 cells (lane 9) and CH-SAH (lane 10). All lysates were probed with a polyclonal rabbit anti-HA antibody (#3425) (ProSci, diluted 1:10,000) and a secondary anti-rabbit antibody (1:20,000). Molecular weight marker is represented on left.
at 130 kDa was observed in lysates from Sf21 cells infected with AcHA:GP64_{\text{FLAG}} (lane 3). The two expected bands at 74 and 65 kDa were detected in the AcHA_{\text{FLAG}} lysate from 72 h.p.i. (lane 4). Both FAdV-HA_{R} and FAdV-HA_{L} had a 95 kDa and a weaker 55 kDa band, and a very faint 60 kDa band as well (lanes 5 and 6, respectively).

FAdV-HA_{R} appeared to have stronger HA expression than FAdV-HA_{L}. AcCHIA_{\text{FLAG}} and AcΔCC had no bands corresponding to HA (lanes 7 and 8, respectively). However a 45 kDa band, observed in Fig 3.12, was also detected for these, and in all other baculovirus-derived lysates (lanes 1-4, 7-8). Wild-type AcMNPV infected cell lysate at 72 h.p.i. also showed a 45 kDa band (lane 11). This is suggestive of cross-reactivity between the commercial polyclonal anti-HA antibody and a protein from baculovirus infected cells. No bands were detected for the mock infected CH-SAH or Sf21 cells (lanes 9-10).

3.3.3 Detection of HA from the recombinant baculoviruses with monoclonal anti-H5 HA antibodies

Recombinant baculovirus-expressed H5 HA was also monitored for its ability to react with monoclonal anti-H5 HA antibodies. Initially a panel of six monoclonal antibodies against H5 HA, kindly provided by Dr. Robert Webster and Bindumadhav Marathe (St. Jude’s Children’s Research Hospital, TN), were assessed for detection of HA_{\text{FLAG}} at 72 h.p.i. AcHA_{\text{FLAG}} and FAdV-HA samples were alternately loaded along the gel, and following immunoblotting, the PVDF membranes were sliced so that each strip included one lane for AcHA_{\text{FLAG}} and the second for FAdV-HA_{R}. Each membrane strip was incubated with different monoclonal antibodies (1:500 dilution). Based on this Western blot, VN04-2, one of the six antibodies tested, showed the strongest reaction and it was selected for further studies.
Figure 3.14 (A) Western immunoblot of lysates of Sf21 cells infected with each recombinant HA-expressing baculovirus. Two µg of each sample was loaded and probed with primary murine monoclonal anti-HA antibody, VN04-2 (1:500) and a secondary anti-mouse HRP conjugated antibody (1:10,000). Lanes 1-4 contained lysates from AcVP39:HA\textsubscript{FLAG}, AcHA:VP39\textsubscript{FLAG}, AcHA:GP64\textsubscript{FLAG} and AcHA\textsubscript{FLAG}, respectively. Lanes 5-6 contained lysates from FAdV-HA\textsubscript{R} and FAdV-HA\textsubscript{L}, respectively. Lanes 7-11 were controls, including lysates from: AcCHI\textsubscript{A\textsubscript{FLAG}} (lane 7), FAdV-9Δ4 (lane 8), AcΔCC (lane 9) and mock-infected CH-SAHA or Sf21 cells (lanes 10 and 11, respectively). Molecular weight marker is represented on the left side of the figure.
Cells infected with each recombinant HA expressing baculovirus were harvested at 72 h.p.i. and probed with mAb VN04-2 at a dilution of 1 in 500 (Fig 3.14). VN04-2 positive HA bands were detected for HA:GP64\textsubscript{FLAG} at 130 kDa (lane 3) and HA\textsubscript{FLAG}, at 74 kDa and a faint one at 25 kDa (lane 4). No bands were detected for either of the VP39 display viruses (lane 1 and 2, Fig. 3.16). Only a faint band was detected for FAdV-HA\textsubscript{R}, while none were detected for FAdV-HA\textsubscript{L} (lanes 5 and 6, respectively). There were no detectable bands in any of the controls, including AcCHIA\textsubscript{FLAG} (lane 7), FAdV-9Δ4 lysate (lane 8), AcΔCC (lane 9) and mock-infected CH-SAH or Sf21mock cells (lanes 10 and 11, respectively). This Western blot indicated that the anti H5 HA monoclonal antibody VN04-2 detected only the overexpressed or GP64-displayed HA but neither of the VP39 displayed HAs.

3.3.4 Detection of HA in Sf21 cells infected with recombinant HA-expressing baculoviruses with polyclonal anti-serum to H5N1 virus

A colorimetric Western blot was conducted to determine whether HA from lysed Sf21 cells infected with the recombinant HA-expressing baculoviruses was recognized by chicken-sera from H5N1 infected birds as the primary antibody, kindly provided by Dr. Robert Webster and Bindumadhav Marathe at St. Jude’s Children’s Research Hospital, (Memphis, TN) (Fig 3.15). This anti-sera reacted with a 104 kDa band for both C and N terminal capsid displayed HA, VP39:HA\textsubscript{FLAG} and HA:VP39\textsubscript{FLAG} (names shown above lanes), 130 and 90 kDa bands for HA,GP64\textsubscript{FLAG}, and 74 and 65 kDa bands for HA\textsubscript{FLAG}. Bands at 64 kDa corresponding to full-length HA were detected for both FAdV-HA\textsubscript{R} and FAdV-HA\textsubscript{L} samples along with some lower molecular weight bands. Additionally, these FAdV-HA samples had a lower mass 37 kDa band,
Figure 3.15 Colorimetric Western immunoblot for Sf21 cells infected with each recombinant HA expressing virus, probed with a H5N1 specific polyclonal Ab (1:500). All samples were probed with a secondary anti-chicken AP conjugated antibody (1:1,000). M denotes the marker protein lane. FAdV-HA (R and L, respectively) infected CH-SAH cells were also used. Cells infected with AcCHIA_{FLAG} (Sf21 cells) mock infected CH-SAH cells, and Sf21 cells were employed as controls. M is molecular weight marker.
corresponding to the HA$_1$ subunit of HA. Some minor bands were detected for CHIA$_{\text{FLAG}}$, mock-infected CH-SA3H and Sf21 cells, (lanes 7, 8 and 9, respectively).

3.3.5 Testing of serum from FAdV-HA vaccinated birds using HA$_{\text{FLAG}}$ as antigen

Having established that known HA antibodies reacted with HA$_{\text{FLAG}}$, sera from FAdV-HA vaccinated birds were tested for the presence of anti-HA antibodies. For this a Western blot was conducted with HA$_{\text{FLAG}}$ protein from infected cells collected at 72 h.p.i. and using serum from chickens vaccinated with FAdV-HA$_R$. The preimmune sera were the negative control. Alkaline phosphotase conjugated anti-chicken IgG was the secondary antibody. A 65 kDa band, corresponding to HA$_{\text{FLAG}}$ was detected for the sera from the vaccinated chickens, but no 65 kDa bands were detected using preimmune sera (Fig 3.16).

3.4 Biological activity of HA expressed in the insect cells

The various recombinant viruses expressed HA as shown by anti-FLAG antibody in Western immunoblots. The HAs also reacted with different monoclonal and polyclonal antibodies raised against H5 specific HA. In addition, the biological activity of the recombinant HA was assessed by hemagglutination and hemadsorption assays and by syncytia formation of infected cells.

3.4.1 Hemagglutination assays

One of the biological activities of HA present in influenza virus virions is that it can mediate hemagglutination of RBCs. The hemagglutinating activity of both sonicated and whole Sf21 cells infected with each of the recombinant HA-expressing baculoviruses, as well as
Figure 3.16 (A) Colorimetric Western immunoblot of Sf21 cells infected with AcHAFLAG (lanes 1-5) and at 72 h.p.i., and probed with serum from chickens vaccinated with the FAdV-HA vaccine as the primary antibody (+ve) (1:500) (lanes 1-3), or with naive chicken serum (-ve) (1:500) (lanes 4 and 5). All samples were probed with a secondary anti-chicken AP conjugated antibody (1:1,000). (B) Schematic of HAFLAG construct that comprises the recombinant HA overexpressed HAFLAG. MW is molecular weight marker.

Chicken sera as primary Ab:
+ve= vaccinated with FaDV-HAR
-ve = FAdV preimmune sera
recombinant budded virions of display viruses, was assessed. Sonicates were used over lysates for the hemagglutination assays to ensure that the detergent did not interfere with the test. Approximately 1x10^6 Sf21 cells were infected with one of each of the recombinant HA-expressing baculoviruses. Cells infected with AcCHIA_FLAG, a non-HA recombinant baculovirus, and AcΔCC and non-infected Sf21 cells were also assessed. Cells were infected at an MOI of 10, collected at 48 h.p.i., and pelleted by centrifugation. Cells were then resuspended in 1 ml of PBS (pH 6.2) and disrupted by sonication. Total sonicate was then quantified and 50 µl was added to the first well of the microtitre plate, and the assay was done as described in Materials and Methods. Purified Newcastle Disease Virus, which has hemagglutinating activity, was the positive control. Although low, hemagglutination was detected for sonicates for AcHA_FLAG, which had an HA titre of 4, and AcHA:GP64_FLAG, which had an HA titre of 2 (Fig 3.17A). Infected Sf21 cells with all other viruses including AcHA:VP39_FLAG, AcVP39:HA_FLAG, AcCHIA_FLAG and AcΔCC showed no evidence of hemagglutination.

In the absence or low levels of hemagglutination of cell sonicates, the hemagglutinating activity of whole (non-sonicated) infected cells was also assessed (Fig 3.17B). Cells were infected with each recombinant HA-expressing baculovirus at an MOI of 10. At 48 h.p.i. infected cells were collected and counted using a hemocytometer. Approximately 3.5 x 10^5 cells from each infection (diluted in PBS) were added to the first well of a 96-well microtitre plate and diluted two-fold across the plate. Cells infected with the HA_FLAG overexpression virus had an HA titre of 8. Those by AcHA:GP64_FLAG had an HA titre of 4, and HA:VP39_FLAG and VP39:HA_FLAG had HA titres of 2 and 0 respectively, although VP39:HA_FLAG showed some evidence of hemagglutination in the first well. The NDV preparation had an HA titre of 2048 while AcΔCC-infected Sf21 cells had a titre of 0.
Figure 3.17 Hemagglutination of virus-infected cells. Cells were infected with each recombinant virus as indicated to the left of each row and collected at 48 h.p.i. Samples were added to the first well of a 96-well plate, and diluted two-fold across the plate. $3.5 \times 10^5$ cells were either sonicated (A) or added to the plate without sonication (B).
Because surface and capsid display involved the direct fusion of HA to baculovirus envelope and capsid proteins, the hemagglutinating ability of budded virions of the HA:GP64\textsubscript{FLAG}, VP39:HA\textsubscript{FLAG} and HA:VP39\textsubscript{FLAG} was also determined. Medium from Sf21 cells infected at an MOI of 10 was collected at 96 h.p.i. and the viruses were concentrated. 100 µl of concentrated viruses were added to the microtitre plate and diluted two-fold. There was no hemagglutination by the recombinant budded virions (data not shown).

3.4.2 Hemadsorption assays

Since cells with biologically active HA in their membranes are capable of adsorbing red blood cells, hemadsorption assays were conducted to determine whether Sf21 cells infected with the recombinant HA-expressing baculoviruses also demonstrated this biological activity.

Hemadsorption was not observed for mock infected cells (Fig. 3.18D) or AcCHIA(ASD)\textsubscript{FLAG} infected cells, suggesting that Sf21 cells or the FLAG tag on its own do not promote hemadsorption. AcΔCC infected cells showed no evidence of hemadsorption, confirming that wild-type baculovirus is not capable of adsorbing RBCs. Cells infected with AcHA\textsubscript{FLAG} (Fig 3.18 B) and AcHA:GP64\textsubscript{FLAG} (Fig 3.18 C) were capable of adsorbing RBCs, but cells infected with AcHA:VP39\textsubscript{FLAG} or AcVP39:HA\textsubscript{FLAG} were not. Both the number of RBCs/cell and the number of cells with adsorbed RBCs increased with the MOI and with time p.i., up to 60 h.p.i. Hemadsorption was not detected in cells infected at an MOI of 1 with any of the recombinant viruses at 20 hours. However, at 40 and 60 h.p.i., hemadsorption was observed in cells infected with either AcHA\textsubscript{FLAG} or AcHA:GP64\textsubscript{FLAG}. When cells were infected at an MOI of 10 with either AcHA\textsubscript{FLAG} or AcHA:GP64\textsubscript{FLAG}, hemadsorption was apparent earlier, at 20 h.p.i., when compared to the earliest detection at 40 h.p.i. for cells infected at a MOI of 1.
A) AcΔCC infected Sf21 cells

B) AcHA\textsubscript{FLAG} infected Sf21 cells
3.18 Hemadsorption assays. Sf21 cells infected with one of each recombinant viruses at an MOI of 1. At 60 h.p.i. 1% RBCs were added to the monolayer of cells infected with (A) AcHA$_{\text{FLAG}}$ (B)
AcHA\textsubscript{GP64\textsubscript{FLAG}} (C) Ac\textDelta CC or (D) Mock infected Sf21 cells. A magnified image of a hemadsorbed (B) and (C), or non adsorbed cells (A) is depicted in the lower right corner of each panel.
Sf21 cells infected with AcHA:VP39\_\text{FLAG} or AcVP39:HA\_\text{FLAG} showed no evidence of hemadsorption, even at 60 h.p.i.

3.4.3 Syncytia formation in Sf21 cells infected with recombinant HA- expressing baculoviruses

It appeared that some of the HA recombinant baculovirus infected cells showed evidence of hemifusion and syncytia formation. This occurred for cells that were infected with AcHA\_\text{FLAG} and AcHA:GP64\_\text{FLAG} where HA would be transported to the membrane, but not AcHA:VP39\_\text{FLAG} or AcVP39:HA\_\text{FLAG} where HA would remain intracellular. For a more in-depth analysis, cells infected with each of the recombinant viruses at an MOI of 10 were monitored for syncytia formation. Control cells included AcΔCC infected, and mock infected cells. Cells infected with AcHA\_\text{FLAG} and AcHA:GP64\_\text{FLAG} showed evidence of syncytia (Fig. 3.19). This suggests that the HA was present on the infected Sf21 cell membrane and was biologically active, being cleaved and mediating fusion with adjacent cells. Syncytia formation was not observed in cells infected with either AcHA:VP39\_\text{FLAG} or AcVP39:HA\_\text{FLAG} nor in AcΔCC infected or mock infected Sf21cells. Since biologically active influenza virus HA mediates syncytia formation between infected cells, this result suggests that HA is present on the membranes of cells infected with AcHA\_\text{FLAG} and AcHA:GP64\_\text{FLAG}, but not those infected with AcHA:VP39\_\text{FLAG} or AcVP39:HA\_\text{FLAG}. 
Figure 3.19 Syncytia formation of AcHA\textsubscript{FLAG} and AcHA\textsubscript{FLAG}GP64 infected cells compared to AcCHI\textsubscript{FLAG} and mock infected cells. Cells were infected at an MOI of 10 and examined using a phase contrast light microscope at 72 h.p.i.
3.5 Neuraminidase expression

A second major membrane protein of the influenza virus is the immunogenic, sialic acid degrading enzyme neuraminidase. Birds infected with influenza virus develop antibodies against both HA and NA. Thus NA was overexpressed in baculovirus for potential use as an alternate diagnostic reagent. The following experiments were conducted with the assistance of an undergraduate project student, James Ackford, who worked under my direction.

3.5.1 NA expression

NA from an H5N1 virus was cloned into a baculovirus vector under the polyhedrin promoter in a similar manner to HA_{FLAG}, except that NA was tagged with the short (9 amino-acid) HA tag (N-YPYDVPDYA-C). Accuracy of cloning was confirmed by PCR analysis using M13 forward and reverse primers, and the cloned region of NA_{HA} was sequenced.

The temporal expression of NA_{HA} was examined via Western blot analysis using a murine anti-HA epitope primary and murine secondary antibody. Sf21 cells were infected with AcNA_{HA} at an MOI of 10, collected from 48 to 96 h.p.i., pelleted and lysed with NP40 lysis buffer. Two µg of lysate from each time point was analyzed.

NA_{HA} was detected at all time points tested at the expected size of 60 kDa (Fig. 3.20). As the course of infection increased, so did the relative amount of the expressed NA_{HA} (Fig 3.20). A 38 kDa band was detected for AcCATH_{HA} infected Sf21 cell lysate reflecting the size of CATH_{HA} and confirming that the anti-HA tag antibody was capable of detecting an HA-tagged protein. No bands were detected for cells infected with AcCHIA(ASD)_{FLAG} or for mock-infected cells.
3.5.2 Neuraminidase assay

The neuraminidase activity of the expressed NA was measured using Amplex® Red Neuraminidase (Sialidase) Assay Kit. A negative control containing only reagents and buffers (no NA) was used as blank, and the A$_{560}$ was subtracted from all samples. Sf21 cells infected with AcNA$_{HA}$ were collected at 96 h.p.i., and following the reaction, A$_{562}$ values were determined and compared to the two positive controls, NA from *Clostridium perfringens*, and 10 μM H$_2$O$_2$, which is a by-product of the reaction. All samples were tested in triplicate, and the mean values are presented in Figure 3.21 (see appendix for raw data). The NA$_{HA}$ whole cell lysate from an undiluted sample had an average A$_{560}$ of 0.9, compared to 0.23 for *Clostridium perfringens* NA and 0.5 for H$_2$O$_2$. Undiluted lysates from AcCHIA$_{FLAG}$ had a mean A$_{560}$ of 0.13 (Fig 3.21).
3.20 Western immunoblot of Sf21 cell lysate from cells infected with AcNA$_{HA}$ from 48 to 96 h.p.i. as indicated above each lane. Molecular weight is represented on the left side of the figure. Two µg of each lysate was probed with a primary murine anti-HA antibody diluted at 1:20,000, and a secondary anti-mouse HRP-conjugated antibody (1:10,000). AcCHIA$_{FLAG}$ and AcΔCC were HA antibody controls. Mock-infected Sf21 cells also served as a negative control.
Figure 3.21 Neuraminidase activity of the recombinant NA was measured by the $A_{560}$ of resorufin, the product of the reaction. A blank reading was taken of wells that contained only reaction buffer and no NA. The $A_{560}$ for the blank was subtracted from all samples and controls. Following incubation in reaction buffers for 30 minutes, absorbance readings for AcNA$_{HA}$ whole cell lysate at 96 h.p.i. was compared to a NA positive control, from *C. perfringens* and a 20 μM solution of H$_2$O$_2$, a byproduct of the reaction. A recombinant baculovirus, AcCHIA(ASD)FLAG, was used as a negative control. Error bars represent the mean standard deviation for each individual trial.
Chapter 4: Discussion

The goal of this study was to construct four baculovirus expression vectors based on: protein overexpression, surface display and capsid display for the production of HA and to assess and compare the relative yield of HA and their utility as antigen to detect anti H5 Abs. All four recombinant viruses were generated and the constructs were confirmed by sequence analysis.

Growth of the viruses was compared to ensure that it was not compromised by insertion of the various HA gene ORF constructs. All four viruses had similar growth kinetics, as expected, since replication of recombinant viruses generated via baculovirus overexpression is typically comparable to that of wild-type AcMNPV (Oker-Blom et al., 2003; Luckow et al., 1993). Furthermore, high-titre recombinant viruses have been produced when foreign proteins are displayed on either the baculovirus surface or capsid (Ernst et al., 1998; Ernst et al., 2000; Molinari et al., 2011; Song et al., 2010). These results confirmed that budded virus production was not compromised by the expression of HA via overexpression, surface display or capsid display. Further, these results implied that any differences observed in HA expression, reactivity, or biological activity among these recombinant viruses would not be due to poor or delayed budded virus production.

The most commonly employed expression method assessed for HA production was protein overexpression. Overexpression of influenza virus HA via BEVS is conceptually not new, and was first described by Kuroda et al. (1986) in which HA was expressed in insect cells and was demonstrated to maintain its biological activity. Since then, numerous studies have been conducted in which HA has been expressed in baculovirus and employed in vaccine development or in diagnostics (Johansson, et al., 1999; Brett et al., 2005; Tao, et al., 2009; Prel
et al., 2007; Wang et al., 2006; Bright et al., 2007; Pushko et al., 2005). Further, a patented and soon-to-be commercially available baculovirus-based seasonal influenza virus vaccine has demonstrated success in ongoing clinical trials (Wang et al., 2006).

4.1 Characterization of expressed HAs using an anti-FLAG antibody

In the present study, HA derived from HPAI A/chicken/Hubei/489(H5N1) was overexpressed using a commercially available BEVS, and protein expression was monitored temporally in both Sf21 and Hi-Five™ insect cells. The expected size of the full-length, unprocessed protein H5 HA₀ was 65 kDa, corresponding to the full-length HA₀ and the carboxy terminal FLAG epitope tag, which was added to the HA to allow for detection. Further, because HA₀ can undergo proteolytic cleavage by furin proteases (Klenk et al., 1975) into HA₁ and HA₂ subunits with masses of 37 and 27 kDa, respectively, it was predicted that there would be two distinct products on the Western blot, of 65 kDa, for the full length HA₀, and 27 kDa, corresponding to the HA₂ subunit including the FLAG tag. The HA₁ subunit was not expected to appear on the blot because the FLAG tag was fused to the C-terminus of the protein, meaning that the 37 kDa HA₁ would not be detected upon cleavage of HA. SDS-PAGE and Western immunoblot (Figures 3.1- 3.4) revealed the two bands using an anti-FLAG antibody from 48 to 96 h.p.i. in both Sf21 and Hi-Five™ insect cells. The 65 kDa band was suggestive of the full-length, unprocessed HA_FLAG fusion. It is probable that the 25 kDa band, though ~2 kDa less than the expected size, corresponds with the HA₂ cleavage product, fused to the C-terminal FLAG tag. These results confirmed that HA_FLAG was expressed as H5 HA protein by AcHA_FLAG, and correlated with previous studies which found that HA undergoes cleavage in insect cells. For instance, Kuroda et al. (1986) reported that when probed with an anti-HA antibody, three bands, corresponding to HA₀ (65 kDa), HA₁ (37 kDa) and HA₂ (27 kDa) were detected. An additional
strongly expressed band at 74 kDa was detected on the Western blot and suggested that further elucidation of the source of this protein was necessary.

Throughout the replication cycle greater HA expression was observed in Hi-Five™ cells than in Sf21 cells. For instance, even when only 5 µg of total protein was loaded into the SDS-polyacrylamide gel, the intensity of the HA\textsubscript{FLAG} bands in Hi-Five™ cells was greater than HA\textsubscript{FLAG} bands in Sf21 cells when 10 µg of protein was loaded. This is consistent with other studies showing that expression in Hi-Five™ is greater than in Sf21 cells (Grandos et al. 1994; Davis et al., 1993; Zhang et al., 2008). Further, Krammer et al. (2010) demonstrated that expression of H5N1 HA (for the development of a virus-like particle–based vaccine) was greater in Hi-Five™ cells than in Sf21 cells, in both the budded virions and infected cellular extracts (Krammer et al., 2010). They concluded that Hi-Five™ cells represent the most advantageous, cost-effective and viable option for the production of large amounts of recombinant baculovirus-derived proteins, particularly if the ultimate goal is vaccine development (Krammer et al., 2010). Despite these results, Sf21 cells were employed more frequently throughout this research, because of their ease of use. Further, Hi-Five™ cells are more easily maintained in serum free medium, which is more costly than serum-containing medium. Because one end goal of this research was to develop an economical diagnostic reagent, we wanted to be cognisant of cost at each point throughout the research.

We compared the recovery of HA\textsubscript{FLAG} in NP40 lysis buffer with a RIPA lysis one for possible application in ELISA. RIPA contains sodium deoxycholate which functions to solubilise membranes, while maintaining the integrity and biological activity of the proteins, ultimately making it an ideal candidate antigen in ELISAs. There was only a marginal difference in the extent of detection of HA\textsubscript{FLAG} between RIPA and NP40 based buffers at 72 h.p.i. in which the
HA\textsubscript{FLAG} band appeared stronger for RIPA buffer. Otherwise, HA recovery seemed comparable between NP40 and RIPA lysis buffers. These results suggest that RIPA buffer would be efficient for cell lysis and solubilisation of HA.

In these studies two bands at 74 kDa and 65 kDa were consistently detected. In previous Western blots using either Sf21 or Hi-Five\textsuperscript{TM} cells grown in the presence of serum and infected with AcHA\textsubscript{FLAG}, the highest molecular weight band at 74 kDa was detected earlier, at 48 h.p.i. In comparison, it was not detected in the soluble fraction of Hi-Five\textsuperscript{TM} cells grown in serum-free medium until 96 h.p.i. in Fig 3.6, regardless of the lysis buffer employed. This difference in timing was unexpected, raising questions about the nature of both the 74 kDa and 65 kDa proteins.

I had two hypotheses regarding the nature of the 65 kDa and 74 kDa bands. The first was that the slowest migrating, 74 kDa band actually represented the full-length HA\textsubscript{FLAG}, and that the 65 kDa band was a cleavage product due to protease activity derived from foetal bovine serum. While bands of 74 kDa and 65 kDa would not correspond with natural HA cleavage, in which bands at 65 kDa and 27 kDa would be expected, it is possible that HA undergoes cleavage by a protease other than furin at a different cleavage site of the HA protein. While research on protease containing-serum is limited, one group found that when expressed in cells grown in the presence of FBS-containing medium, recombinant human hepatocyte growth factor (r-HgGF) underwent cleavage (Shimomura et al., 1992). This cleavage was not observed when cells were grown in serum free medium. Researchers identified and purified a novel serine protease present in FBS, and when they added this protease to serum free medium, cleavage of r-hHGF was noted (Shimomura et al., 1992). While H5 HA undergoes cleavage by furin proteases and not by serine
proteases, this research provides evidence that proteases in serum can alter the cleavage pattern of various proteins.

My second hypothesis was that the 65 kDa band represented the full-length non glycosylated HA\textsubscript{FLAG} and the 74 kDa band corresponded to a glycosylated version of HA\textsubscript{FLAG}. HA undergoes several post-translational modifications, including formation of disulfide bonds, (Chreighton et al., 1988; Segal et al., 1992), addition of N-glycosidic oligosaccharide side chains (Keil et al., 1985) and acylation (Schmidt et al., 1982). Glycosylation of HA is a possible source for the higher 74 kDa band of HA\textsubscript{FLAG} observed in immunoblots. Since each N-linked glycosylation adds approximately 2.5 kDa to a protein (Kornfeld et al., 1985), and it has been well documented that HA undergoes significant N-linked glycosylation to its HA\textsubscript{1} subunit, it is possible that that 65 kDa band corresponds to non glycosylated HA while the 74 kDa band corresponds with the processed, full-length version of HA. This hypothesis is consistent with other reports in which recombinant HA produced via BEVS remained biologically functional and properly glycosylated (Wang et al., 2006).

The hypothesis that proteases present in FBS were responsible for the 74 kDa and 65 kDa bands was investigated first. When expression of HA\textsubscript{FLAG} in Hi-Five\textsuperscript{TM} cells was compared in the presence or absence of serum containing medium, both the 74 kDa and 65 kDa bands were present in both samples at similar levels (Fig 3.6). Expression of HA\textsubscript{FLAG} was marginally higher in cells grown in the presence of FBS than in the absence of FBS. It was expected that if proteases in FBS were responsible for cleavage, then the 74 kDa protein would undergo greater cleavage in infected cells grown in the presence of serum. Alternatively, there should have been a single, stronger band in infected cells grown in the absence of serum. However, the results were the opposite with the levels of the 74 kDa band in cells grown in the presence of serum
being higher. This led to the conclusion that FBS is not responsible for cleavage and for the presence of the 74 and 65 kDa bands. This experiment was repeated in Sf21 cells and the result was the same (data not shown). While some have found that serum enhances cleavage, others have reported that cleavage activity is actually enhanced in the absence of serum (Gardner et al., 1994), as many sera contain protease inhibitors. This may provide an explanation as to why expression of the 74 kDa protein for HA\textsubscript{FLAG} was slightly greater in infected cells grown in the presence of serum. This result led me to investigate whether glycosylation was responsible for the observed 74 and 65 kDa proteins from Sf21 and Hi-Five\textsuperscript{TM} cells infected with AcHA\textsubscript{FLAG}.

To determine if the 74 kDa protein is a glycosylated form, AcHA\textsubscript{FLAG} was grown in the presence or absence of tunicamycin (Fig 3.7). In the presence of tunicamycin, a known inhibitor of glycosylation (Duksin et al., 1982), the 74 kDa protein was absent while the 65 kDa protein remained, suggesting that the 74 kDa protein resulted from glycosylation of the 65 kDa HA. Because the difference in size is about 10 kDa, it can be inferred that four sites of the HA protein were glycosylated. This concurs with other reports suggesting that the HA protein is heavily glycosylated, with H5 and H7 subtypes having anywhere from 4-6 glycosylation sites (Matrosovich et al., 1999). While some glycosylation sites are conserved in the H5 subtype, such as at position 161 (Matrosovich et al., 1999), others vary (Matrosovich et al., 1999; Baigent et al., 2001). Interestingly, while the H5 and H7 subtypes of HPAI viruses that infect poultry have up to four (Banks and Plowright, 2003), this is not necessarily correlated with increased pathogenicity (Banks and Plowright, 2003). Regardless, one of the main advantages of BEVS is that it allows for the production of fully processed protein that is post translationally modified in the insect cells (Luckow et al., 1995). My results are consistent with previous reports that baculovirus expressed HA has at least two distinct bands on a Western blot, and that in the
presence of PNGase F, an enzyme which cleaves N-linked glycans, and endoglycosidase H, an enzyme that cleaves oligosaccharides, the upper band disappears (Wang et al., 2006). These results clearly show that glycosylation of the HA protein occurs when it is overexpressed via BEVS making it an attractive candidate for production of immunologically reactive HA.

GP64 display represents a relatively new advancement in the field of baculovirus expression yet it has already been extensively described in the literature (Ernst et al., 2000; Grabherr et al., 2001; Jin et al., 2008; Boublik et al., 1995). Here, HA_{FLAG} was fused to the N-terminus of the baculovirus GP64 protein. The expected 129 kDa band was observed in both Sf21 and Hi-Five™ insect cells when probed with the anti-FLAG and anti-GP64 antibodies (Fig 3.8). Additionally, a smaller 90 kDa band was present on the blots. This band corresponds with only the HA_{2} subunit and FLAG-fused epitope tag to the GP64 protein, suggesting that GP64 fusion can produce some proteolytically cleaved HA. Finally, because wild-type GP64 was retained in the bacmid, a 64 kDa band was observed using the anti-GP64 antibody but not the FLAG antibody. While surface-displayed H5 HA has been described (Jin et al., 2008; Yang et al., 2006), my work is the first to include the HA signal peptide rather than the GP64 signal peptide for translocation to the ER (Jin et al., 2008; Yang et al., 2006). Retaining the native signal peptide of a glycoprotein for translocation has been shown to be superior to replacing it. For example, when the signal peptide of Lassa virus glycoprotein GP-C was substituted with the signal peptides of influenza virus HA or CD8, proteolytic cleavage of GP-C into GP-1 and GP-2 did not occur (Eichler et al., 2003).

Recently, Yang et al. (2006) conducted a study which compared the effect of employing only small domains from the GP64 protein, such as the signal peptide and cytoplasmic domain, for fusion of HA rather than the entire full length GP64 protein. Interestingly, they found that
when HA was fused between the N-terminal GP64 signal peptide and the C-terminal cytoplasmic domain of GP64, while omitting the rest of the GP64 protein, HA was sufficiently expressed on the envelope of the virion. For this fusion, the GP64 TM domain was removed, and instead the HA TM domain was employed for translocation to the membrane. The major difference between this fusion, which employed only small portions of the GP64 protein, and the HA:GP64\textsubscript{FLAG} produced in my work is the size of the entire GP64 protein plus HA\textsubscript{FLAG}. The HA:GP64\textsubscript{FLAG} fusion produced in my work was over 130 kDa, whereas the fusion produced by Yang et al (2006), comprising only the GP64 signal peptide, HA (minus its 16 amino acid signal peptide), and the GP64 CTD, was not much larger than the HA itself. This smaller fusion may better provide for proper folding of HA, or budding of the virus, and therefore future experiments could compare these two approaches.

VP39 display is the most novel form for baculovirus expression (Kukkonen et al., 2003). VP39 is not a glycoprotein and therefore is not directed to the ER and Golgi apparatus but remains cytoplasmic and nuclear. Hence the HA transmembrane C-terminal domain and N-terminal signal peptide were removed to ensure that the VP39 fusion proteins remained intracellular and could be incorporated into viral nucleocapsids. The predicted size of each VP39 fusion was 104 kDa based on the amino acid sequence. Both the anti-FLAG antibody and the anti-VP39 antibody revealed the expected 104 kDa protein in cells infected with either the HA:VP39\textsubscript{FLAG} or VP39:HA\textsubscript{FLAG} fusion viruses (Fig 3.9). The anti-VP39 antibody detected the same 104 kDa HA, as well as a 39 kDa band corresponding to the wild-type VP39 protein, which was retained in the bacmid so that virus production would not be compromised. Also noteworthy is that C-terminal VP39 (VP39:HA\textsubscript{FLAG}) display was expressed at higher levels in both Sf21 and Hi-Five\textsuperscript{TM} cells than N-terminal VP39 (HA:VP39\textsubscript{FLAG}) display. My data are in accordance with
the reports of Kukkonen et al. (2003), that HA can be successfully fused to either end of the VP39 protein and that removal of the SP and TM does not affect detection by the anti-FLAG or anti-VP39 antibodies. Further, my data indicated that C-terminal VP39 display may promote greater expression of the foreign protein than N-terminal VP39 display.

A notable difference between capsid display and the other two expression methods was the lack of a cleavage product by the VP39 display. If HA cleavage occurred, then 77 kDa and 66 kDa bands should have been present on the blots for HA:VP39FLAG and HA:VP39FLAG, respectively. For the C-terminal fusion, the cleavage product would correspond to VP39, FLAG and the HA1 subunit (37 kDa) because the HA protein was tagged with FLAG at its N-terminus. Alternatively, the cleavage product for the N-terminal fusion would correspond to VP39, the HA2 subunit, and FLAG, as the HA protein was tagged with FLAG at its C terminus. However, because HA undergoes cleavage by furin proteases, which are present in the trans-Golgi network of the cell (Stieneke-Grober et al., 1992), and due to the fact that the SP was removed from HA preventing it from being directed to the ER/Golgi apparatus, cleavage of HA0 was not expected in VP39 display. Regardless, it is possible that lack of cleavage of HA0 could affect its biological activity.

One of the most unique attributes of capsid display is that the VP39 protein tolerates fusions to both its C and N termini (Kukkonen et al., 2003). Kukkonen et al. (2003) showed that fusion to either the amino or carboxy terminus of VP39 allowed for sufficient display of foreign protein (EGFP) on the baculovirus capsid. My thesis research is the first to apply VP39 display to a viral protein, specifically the HA of influenza virus, corroborating previous research by Kukkonen et al. (2003), and two others conducting capsid display by fusing their protein of interest to the N-terminus of VP39 (Song et al., 2010; Molinari et al., 2011). Further, because
the present results suggest that C-terminal VP39 display may yield more recombinant protein, future researchers should consider employing C-terminal, rather than N-terminal VP39 display, particularly when trying to produce large amounts of foreign protein.

One of the main advantages of baculovirus display is that it allows for localization of the protein of interest directly on the budded virion, (Kukkonen et al., 2003; Luckow et al., 1995; Oker-Blom et al., 2003; Song et al., 2010; Molinari et al., 2011). My research has confirmed that recombinant baculovirus produced HA is detectable in both Sf21 and Hi-Five™ insect cells, regardless of the expression method used. Not surprisingly the concentrated AcHAFLAG budded virions also contained HAFLAG. Because HA on its own goes to the cell membrane, it is possible that the baculovirus trapped some HA in the envelope of its virion as it buds from the cell. The HA in either HAFLAG or HA:GP64FLAG virions was present at consistently higher levels than in the capsid display virions. While the hypothesis that GP64 displayed HA expression would be strongest was confirmed by these results, the prediction that capsid displayed HA, particularly at the C-terminus, would produce strong HA expression, was not. Though the majority of research on capsid display to date has been on localization rather than protein expression and recovery, it was shown using both EM and Western immunoblot that capsid display is an effective tool for recovery of the protein of interest from the budded virions (Kukkonen et al., 2003; Molinari et al., 2012). Instead, I was able to demonstrate only a very faint HA-specific band for both capsid-displayed viruses at the expected size of 104 kDa. In contrast, strong bands corresponding to overexpressed HA were observed for the recombinant budded virions. These results dispute research that baculovirus capsid display is superior to overexpression of membrane bound proteins for the successful generation of budded virions containing the protein of interest.
(Kukkonen et al., 2003). My results suggest that overexpression is an effective method for producing budded virions which contain the protein of interest.

One overarching observation from my results is that HA\textsubscript{FLAG} and HA:GP64\textsubscript{FLAG} appeared to undergo proteolytic cleavage in both Sf21 and Hi-Five\textsuperscript{TM} cells while VP39:HA\textsubscript{FLAG} and HA:VP39\textsubscript{FLAG} did not. In a natural host, influenza virus infection is facilitated by binding of HA to sialic acid receptors on the host cells. Following internalization of the virion by endocytosis, the acidic pH of the cell facilitates fusion of the virion to the endosomal membrane of the cell, allowing viral uncoating. The acidic pH also activates the proteases, such as furin, required for cleavage of HA\textsubscript{0} into HA\textsubscript{1} and HA\textsubscript{2} subunits which is considered a prerequisite for successful influenza virus infection. My results up to this point indicated that HA\textsubscript{FLAG} and HA:GP64\textsubscript{FLAG} underwent cleavage, it is probable that the pH needed for activation of HA (which is about 6) corresponded to the pH of the infected Sf21 cells (6.2) Conversely, because the HA in capsid display viruses did not undergo cleavage, it seems that the pH of activation of HA did not correspond with the pH of the insect cells. Because the pH of insect cells would have been expected to remain consistent at 6.2, mutations to the HA protein (i.e. removal of the signal peptide) and fusion of VP39 might have led to an alteration of the pH of activation of HA\textsubscript{0}. This prediction is consistent with research that shows minor mutations to various regions of HA can lead to significant changes in the required pH for activation (Daniels et al., 1987; Cross et al., 2001, Reed et al., 2009)

Cleavage of overexpressed HA\textsubscript{0} into HA\textsubscript{1} and HA\textsubscript{2} by Sf21 cells was expected, as it has been demonstrated in the literature (Kuroda et al., 1986; Wang et al. 2006; Cox et al., 2008). Additionally, the observed cleavage of HA:GP64\textsubscript{FLAG} by Sf21 cells was expected, as HA:GP64\textsubscript{FLAG} was translocated to the ER and then the membrane of the infected cell. However,
the cleavage of GP64-fused HA shown in my results has not actually been reported in the literature. For instance, when HA was fused between the signal peptide and cytoplasmic domain of GP64 by Yang et al. (2007), cleavage into HA₁ and HA₂ subunits was not observed. Yang et al. (2007) stated that cleavage did not occur because Sf9 cells lack furin, citing a review by Kost and Con dreay (1999). However, it seems that Yang et al. may have misinterpreted Kost and Con dreay because the review explicitly states that a gene homologous to furin has been identified in Sf9 cells by Ceiplik et al. (1998) and further suggests that lower levels of HA cleavage by insect cells compared to mammalian cells could be due to a smaller quantity of furin in insect cells, rather than an absence of it. Based on my research, it seems that furin (or a furin-like protease) is indeed present in insect cells, as the proteins detected by anti-FLAG for HA\textsubscript{FLAG} and HA:GP64\textsubscript{FLAG} corresponded to HA₀ and the natural HA₂ cleavage product. My result is therefore in agreement with Ceiplik et al. (1998), but not Yang et al. (2007) and Jin et al. (2008). Further, because furin is actually a constituent of Sf9 cells, it might be possible that the lack of cleavage of the HA:GP64 fusions reported by Yang et al. and Jin et al. was actually because they used the GP64 signal peptide and not that of native HA.

4.2 Characterization of expressed HAs using anti HA antibodies

The HAs were first tested with the anti-FLAG antibody to confirm that HA was expressed in Sf21 cells infected with each recombinant virus. However the baculovirus-expressed HA should also be recognized by anti H5 HA antibodies. Therefore the HAs were first tested with a commercial monospecific polyclonal anti-H5 HA antibody (ProSci 3425), which was raised in rabbits against a synthetic peptide corresponding to 15 relatively conserved amino acids in the middle of the avian influenza HA protein. Both the glycosylated (74 kDa) and non-glycosylated (65 kDa) forms of AcHA\textsubscript{FLAG} were detected with this antibody from 48 to 96 h.p.i.
and that was similar to the results obtained with the anti-FLAG antibody. The C-terminal HA$_2$ subunit was not detected since the recognition site for the antibody was only on the HA$_1$ subunit. The results of this Western blot suggest that overexpressed HA can be detected by polyclonal anti-HA antibodies generated against an H5 avian influenza virus.

HA bands were also detected for each display virus at the expected sizes with the polyclonal (#3425) antibody. This result implies that the specific amino acid sequence required for detection of HA by this commercial polyclonal antibody was retained on each recombinant HA. HA:GP64$_{\text{FLAG}}$ also reacted with the polyclonal anti-HA antibody as evidenced by bands corresponding to the full-length fusion as well as the 90 kDa cleavage product. This was expected because not only did Western immunoblot using the anti-FLAG antibody against the same lysates yield a positive result, but also that cleavage, and therefore processing of HA, occurred in Sf21 cells infected with AcHA$_{\text{FLAG}}$GP64.

Capsid display viruses also produced the expected bands when probed with the polyclonal anti-HA antibody. While the anti-FLAG antibody detected the full-length fusion in both capsid displayed viruses, no cleavage products were visible, suggesting that processing of HA by Sf21 cells infected with these viruses was impaired. This was expected due to the removal of the HA SP and TM domains in these recombinant constructs, which can alter the pH at which HA activation is triggered (Reed et al., 2009). Due to the lack of cleavage along with the fact that two domains critical for processing and production of HA were removed in the capsid displayed viruses, it was predicted that detection of capsid-fused HA by the polyclonal anti-HA antibody might be impaired. However, this was not the case, and instead, the same 104 kDa band detected by anti-FLAG was detected using anti-HA. Clearly, the epitope sequence required for detection of HA by #3425 remained intact in the capsid-displayed viruses, despite
their observed lack of cleavage in previous Western blots when using the anti-FLAG antibody for detection.

The baculovirus based HAs were also evaluated against several monoclonal anti-H5 HA antibodies. One antibody, VN04-2 showed detectable levels of HA. This monoclonal antibody detected HA from cells infected with either the surface displayed (130 kDa) or overexpressed (75 kDa) viruses. In contrast, VP39:HA\textsubscript{FLAG} and HA:FLAG\textsubscript{VP39} were not detected using this monoclonal antibody. The neutralizing VN04-2 antibody recognizes the lysine, which is an absolute requirement, at the 140s antigenic loop on the HA\textsubscript{1} subunit of the folded globular head of the HA protein (Lim et al., 2008; Kaverin et al. 2007). Previous results have shown that even a single mutation to the lysine codon results in poor or absent reactivity of the antibody to HA. The fact that VN04-2 recognizes an epitope in the HA\textsubscript{1} subunit explains why no HA\textsubscript{2} cleavage products were detected for GP64:HA\textsubscript{FLAG} or HA\textsubscript{FLAG}. Additionally, it seems that VN04-2 is sensitive, reacting with HA only if lysine is present on the globular head region of the HA\textsubscript{1} subunit. Removal of the signal peptide and transmembrane domains of the HA in the VP39 display viruses might alter the folding of HA, concealing the epitope on the protein thereby rendering the protein unrecognizable by the monoclonal antibody.

The difference between the recognition of VP39:HA\textsubscript{FLAG} or HA:VP39\textsubscript{FLAG} by the polyclonal and monoclonal anti-HA antibodies may be due to their specificity. It is likely that the monoclonal antibody recognized properly oriented, processed, folded and cleaved HA, while the polyclonal HA antibody recognized an amino acid sequence that was detectable regardless of folding and cleavage of HA\textsubscript{0} to HA\textsubscript{1} and HA\textsubscript{2} subunits.
Following confirmation that all four recombinant HAs could be detected at the very least by the polyclonal anti-H5 antibody (#3425), all four HAs were used to determine their ability to detect Ab from sera of birds immunized with HA. This chicken serum reacted with all recombinant HAs and bands were seen at the expected sizes in the Western blots. These results are similar to those obtained for the rabbit polyclonal anti H5 antibody in that all four recombinant HAs were detected. This is not surprising as polyclonal antibodies recognize numerous epitopes. These results are encouraging for further investigation into the utility of these HAs as antigens in an ELISA to test for HA specific antibodies in chicken sera.

HA\textsubscript{FLAG} protein was detected by FLAG antibodies, and a variety of antibodies as discussed above suggesting its utility as a potential diagnostic reagent. To this end, I used the HA\textsubscript{FLAG} protein in Western blots to test serum samples from chickens vaccinated with a recombinant fowl adenovirus, FAdV-HA, to see if anti HA antibodies could be detected. A band at 64 kDa was detected which was not seen when preimmune sera were the primary antibody source, indicating the specificity of the reaction. While there were clear reactions, the bands observed were faint (Fig 3.15). One explanation might be that the sera were stored for too long, and the antibodies might have degraded. Additionally, the only available secondary antibody was recommended for use in ELISAs rather than Western immunoblot. While these results are preliminary they suggest that HA\textsubscript{FLAG} could be used to detect H5 specific antibodies in sera from chickens vaccinated with FAdV-HA or other HA containing vaccines. Furthermore, it suggests that that this prototype FAdV-HA vaccine may elicit a sufficient anti-HA antibody response in chickens. Moreover, these results indicate that the baculovirus-derived recombinant HA has potential for diagnostics purposes.
4.3 Biological activity of recombinant HAs

Hemagglutination of red blood cells is a known biological activity of influenza virus HA, (Vyas and Shulman, 1970) and is still a common diagnostic method for influenza virus infection. In the present study, the hemagglutination assays was conducted using either sonicated Sf21 cell extracts or whole Sf21 cells that were infected for 20, 40 and 60 hours, with each recombinant HA-expressing baculovirus. The hemagglutination potential of concentrated budded virions from display viruses was also assessed. Some hemagglutinating activity was observed for sonicates of cells infected by each of the four viruses, but at low levels. Sonicates of AcHA\textsc{FLAG}, infected cells had an HA titre of 4. This result is not consistent with published data which suggests that extracts from cells infected with an overexpressed HA-based baculovirus produce strong hemagglutination with HA titres upwards of 64 (Kuroda et al., 1986).

Whole Sf21 cells infected with the recombinant viruses expressing HA were then tested in hemagglutination assays, and again the highest HA titre was obtained for AcHA\textsc{FLAG} infected cells. While HA titres were slightly higher using whole infected cells, they were still relatively low when compared to those reported by Kuroda et al (1986). The absence of NA in the HA-expressing baculoviruses may contribute to the poor hemagglutinating activity, however other factors may have contributed as well. For example, the MOI used in the Kuroda study is not reported and may have been higher than the 10 that I used.

Another explanation for the poor hemagglutinating ability of infected whole cells is their larger size compared to HA- containing, virions reducing their ability to form a cross linked matrix. Despite having low titres in the hemagglutination assay the results suggest that
AcHA<sub>FLAG</sub> and AcHA:GP64<sub>FLAG</sub> infected Sf21 cells can hemagglutinate red blood cells and that the recombinant HAs are biologically active.

Another biological activity of HA is that it can adsorb red blood cells when exposed on the surface of infected cells. Only cells infected with either overexpressed or surface displayed HA viruses showed hemadsorption. While the level of hemadsorption was more strongly pronounced in cells infected with the overexpression virus, it was also observed in cells infected with the surface displayed virus. These results are consistent with published data (Kuroda, 1989; Wang et al., 2006; Van Wyke et al., 1987; Ernst et al., 1998). It also corroborates the results of the biological activity shown by hemagglutination assays. Not surprisingly cells infected with either capsid-displayed viruses did not show hemadsorption, since the TM domain of HA was removed in these two viruses, and therefore HA was not expected to be embedded in the membranes of infected insect cells.

While suggestive syncytia formation was observed by cells infected with AcHA<sub>FLAG</sub> and AcHA:GP64<sub>FLAG</sub>, the syncytium observed was not typical in that there were no clear multinucleate cells. The literature seems to generally accept that HA mediates full fusion between influenza virus-infected cells (Simpson and Lamb, 1992; Sakai et al., 2002; Melikyan et al., 1995). However, most papers use the terms “full fusion” and “syncytia formation” interchangeably, and many count syncytia formation in influenza virus infected cells as an indicator of full fusion (Simpson and Lamb, 1992; Sakai et al., 2002; Melikyan et al., 1995). While the difference between HA mediated fusion and syncytia formation is not made clear in the literature (Simpson and Lamb, 1992; Sakai et al., 2002; Melikyan et al., 1995), there was an observable fusion occurring between cells infected with AcHA<sub>FLAG</sub> and AcHA:GP64<sub>FLAG</sub>, and not cells infected with AcHA:VP39<sub>FLAG</sub> or AcVP39:HA<sub>FLAG</sub>. Even if this was not a typical
syncytia formation, this result suggests that the fusion-mediating properties of HA were retained only when HA was directed to the membrane of infected cells.

4.4 Neuraminidase Expression

Neuraminidase is another major membrane protein of influenza viruses and thus part of the immune response against influenza viruses is targeted to NA. Therefore baculovirus-expressed NA could also be used for diagnostic purposes. NA, with a C-terminal HA epitope tag to follow its synthesis, was overexpressed via baculovirus. The overexpression of NA, including N1 and N2 subtypes, via BEVS has been well documented (Mather et al., 1992, Pushko et al., 2005, Latham et al., 2001, Bright et al., 2008). A single 60 kDa band, consistent with the size of the native N1 NA, was present on the Western immunoblot, and expression increased over time p.i. This result is consistent with the literature. For instance, Weyer and Possee found that baculovirus expression of NA resulted in the detection of a single 58 kDa band with a monoclonal anti-NA antibody (Weyer and Possee, 1991). A similar experiment was conducted by Mather et al. (1992) and a 60 kDa band corresponding to full-length NA was identified.

The present NA construct was fused with an amino terminal HA epitope tag derived from the influenza virus HA protein. Therefore use of $\text{NA}_{\text{HA}}$ in an ELISA has the potential to detect anti HA antibodies as well as anti NA antibodies. However, the amino acid sequence of the HA-epitope tag was not present in the H5 HA amino acid sequence. This suggests that there will be no reactivity between this HA epitope and anti HA antibodies for example in sera from chickens vaccinated with FAdV-HA.

In addition to its strong expression in the insect cell, as demonstrated by Western immunoblotting, $\text{NA}_{\text{HA}}$ was also found to be enzymatically active. Because it was active in Sf21
cells, it has likely undergone proper post translational modifications and folding, making NA an attractive candidate protein for diagnostic purposes such as one arm of a DIVA. For example, the NA and HA expressing baculoviruses developed here could be used concurrently in separate ELISAs to test a single serum sample to differentiate infected from vaccinated animals. Serum from an individual infected with influenza virus would have positive ELISAs for both HA and NA, while serum from animals vaccinated with an HA-only vaccine would be positive for only HA. To date, the generation of a reliable DIVA strategy has been challenging. Several different types of DIVAs have been developed (Avelleneda et al., 2010; Capua et al., 2002), but none have been readily adopted as part of a standard operational protocol. A DIVA such as the one I described would have specific advantages over the previously developed DIVAs. First, it would be versatile. While it could be used to test sera from chickens vaccinated with an HA-specific vaccine (e.g. FAdV-HA), it could also test sera from chicken vaccinated with an NA-based vaccine. Finally, the scale-up of baculovirus expressed proteins is rapid, efficient and inexpensive and therefore an ELISA based on BEVS expressed proteins may be more readily adopted by governmental regulatory agencies.

4.5 Conclusions and Future Considerations

Several experiments were conducted and baculovirus constructs expressing an H5 avian influenza virus HA generated to determine which expression method is preferable for the production of HA. While HA was detected for each of the recombinant baculoviruses by various antibodies including anti-FLAG and anti-HA, it was biologically active in only the GP64 displayed and overexpression viruses. Further, the recombinant protein expressed by these two viruses were detected by anti H5 HA monoclonal antibody, while capsid displayed viruses were not. Finally, processing of HA by glycosylation occurred in cells infected with AcHAFLAG
(cleavage and glycosylation), and inferred for AcHA:GP64_{FLAG}, while processing in AcVP39:HA_{FLAG} and AcHA:VP39_{FLAG} infected cells was not evident. Finally, HA_{FLAG} and HA:GP64_{FLAG} were found in budded virions, but VP39:HA_{FLAG} and HA:VP39_{FLAG} were not. Taken together, the baculovirus expression methods of overexpression and surface display produced greater amounts of HA than capsid display. Furthermore, the HA was biologically active in terms of hemagglutination and hemadsorption. Thus AcHA_{FLAG} and AcHA:GP64_{FLAG} would be the best choice for production of HA for use as a diagnostic antigen.

Future directions should focus on developing the various HAs for the detection of HA antibodies in ELISA. Essentially we have established that baculovirus-derived HA is detectable by several antibodies including in chicken serum, and that preimmune chicken sera does not react with BV expressed HA. These results support the development of an ELISA to detect anti-HA antibodies present in sera from chickens infected with an H5N1 or vaccinated with an HA based vaccine such as FAdV-HA. If this ELISA proves to be effective at detecting anti-HA antibodies in chickens, then the next step would be to determine whether overexpressed NA is capable of detecting anti NA antibodies in sera from chickens infected with N1 containing influenza viruses but not in sera from chickens vaccinated with an HA derived vaccine. Finally, if both ELISAs are successful, then their utility in a DIVA system should be assessed.

While hemagglutination was observed, it was only at low levels, which was inconsistent with the data in the literature. This could be due to poor optimization of the hemagglutination assay, or incomplete sonication or activation of HA. This assay should be repeated and different types of cell lysis methods should be explored. If trying to optimize this assay is still unsuccessful, then the single radio immunodiffusion assay (SRID) should be considered. The SRID assay measures the interaction between antigen and antibody in an agarose gel, which
produces a precipitation zone that is directly proportional to the amount of antigen added to the gel. Therefore, a reference antigen can be used as a source for comparison and the amount of antigen in an unknown sample, (e.g. HA) can be better quantified. This method has been adopted by the Food and Drug Administration in the United States and is considered a reliable tool for measuring influenza virus antibody response (Williams et al., 1993, Kalbfuss et al., 2008; Kessel et al., 2012). Further, Cox et al. (2008) successfully employed SRID assays using HA produced via BEVS. SRID assays may help to corroborate the evidence presented here that AcHA\text{FLAG} and AcHA:GP64\text{FLAG} are suitable diagnostic antigens.

With regards to the utility of capsid display, the C-terminal fusion of the HA protein was more efficient than N-terminal fusion, and this has not yet been reported in the literature. Therefore, future experiments should try to further elucidate whether C-terminal fusion does indeed result in more foreign protein of interest for other proteins e.g. NA. Additionally, because capsid proteins and membrane proteins differ greatly in their trafficking and processing through infected cells, it is likely that capsid display would be better employed for the expression of a non membrane bound or nor glycosylated influenza virus protein, such as NP. In the cell, NP is involved in packaging and virus assembly in the infected cell, and because it does not go to the membrane, is not trafficked to the ER or Golgi upon virus entry. Further, NP–based vaccines induce heterosubtypic protection against influenza virus in humans, through a cytotoxic T-lymphocyte response (Ulmer et al., 1993), and stimulate strong non-neutralizing antibody response (Carragher et al., 2008). Therefore, capsid displayed NP may be useful in a study of this nature, particularly for the generation of an alternative influenza virus antigen.

The present study examined and compared the effectiveness of three baculovirus expression methods, including capsid display, overexpression and surface display, for the
production of biologically active, highly expressed, HA for use as antigen. The latter two expression methods were found to be most effective for this purpose. The long-term goal of this research is to develop a versatile serological ELISA-based diagnostic assay for influenza virus antibodies such as HA and NA. Such an assay would provide a means to evaluate the ability of a vaccine to generate a detectable antibody response in sera from vaccinated animals, and therefore, help to confirm vaccine response. Together with monitoring for a second influenza virus protein not present in the vaccine, the DIVA assay could be employed for differentiation of vaccinated from infected animals, which could ultimately facilitate a means for expansion of the poultry industry within Canada.
REFERENCES


Banks, J., Plowright, L., 2003. Additional glycosylation at the receptor binding site of the hemagglutinin (HA) for H5 and H7 viruses may be an adaptation to poultry hosts, but does it influence pathogenicity? Avian Dis., 942-950.


Capua, I., Mutinelli, F., 2001. Mortality in Muscovy ducks (Cairina moschata) and domestic geese (Anser anser var. domestica) associated with natural infection with a highly pathogenic avian influenza virus of H7N1 subtype. Avian Pathol. 30, 179-183.


Appendices

Appendix A

Raw data for viral growth curve

Table A1: Individual virus titres for each recombinant HA-expressing virus from individual trials

<table>
<thead>
<tr>
<th>Trial</th>
<th>Virus</th>
<th>12 h.p.i.</th>
<th>24 h.p.i.</th>
<th>36 h.p.i.</th>
<th>48 h.p.i.</th>
<th>72 h.p.i.</th>
<th>96 h.p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AcHA:VP39_FLAG</td>
<td>2.11E+03</td>
<td>9.17E+02</td>
<td>5.45E+05</td>
<td>5.45E+05</td>
<td>1.23E+08</td>
<td>2.34E+08</td>
</tr>
<tr>
<td>2</td>
<td>AcHA:VP39_FLAG</td>
<td>4.88E+03</td>
<td>3.46E+04</td>
<td>3.12E+05</td>
<td>3.12E+05</td>
<td>3.62E+08</td>
<td>9.00E+08</td>
</tr>
<tr>
<td>1</td>
<td>AcVP39:HA_FLAG</td>
<td>1.55E+03</td>
<td>4.99E+03</td>
<td>2.45E+05</td>
<td>2.45E+05</td>
<td>3.68E+08</td>
<td>1.36E+09</td>
</tr>
<tr>
<td>2</td>
<td>AcVP39:HA_FLAG</td>
<td>3.57E+02</td>
<td>4.99E+03</td>
<td>3.49E+04</td>
<td>3.49E+04</td>
<td>3.80E+08</td>
<td>1.36E+09</td>
</tr>
<tr>
<td>1</td>
<td>AcHA:GP64_FLAG</td>
<td>8.59E+02</td>
<td>1.85E+03</td>
<td>5.19E+04</td>
<td>5.19E+04</td>
<td>1.90E+07</td>
<td>2.46E+09</td>
</tr>
<tr>
<td>2</td>
<td>AcHA:GP64_FLAG</td>
<td>2.00E+03</td>
<td>3.50E+03</td>
<td>8.47E+04</td>
<td>8.47E+04</td>
<td>1.30E+07</td>
<td>2.61E+08</td>
</tr>
<tr>
<td>1</td>
<td>AcHA_FLAG</td>
<td>1.68E+04</td>
<td>4.52E+04</td>
<td>2.70E+05</td>
<td>2.70E+05</td>
<td>4.50E+07</td>
<td>5.70E+08</td>
</tr>
<tr>
<td>2</td>
<td>AcHA_FLAG</td>
<td>5.17E+02</td>
<td>4.46E+04</td>
<td>2.70E+05</td>
<td>2.70E+05</td>
<td>3.30E+07</td>
<td>2.08E+08</td>
</tr>
<tr>
<td>1</td>
<td>AcΔCC</td>
<td>2.08E+03</td>
<td>3.31E+04</td>
<td>8.59E+04</td>
<td>8.59E+04</td>
<td>1.10E+09</td>
<td>1.57E+09</td>
</tr>
<tr>
<td>2</td>
<td>AcΔCC</td>
<td>1.01E+03</td>
<td>2.57E+04</td>
<td>4.55E+05</td>
<td>4.55E+05</td>
<td>1.70E+08</td>
<td>4.77E+08</td>
</tr>
</tbody>
</table>

Table A2 Mean virus titres for each recombinant HA-expressing virus

<table>
<thead>
<tr>
<th>Virus</th>
<th>0</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>48</th>
<th>72</th>
<th>96</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcHA:VP39_FLAG</td>
<td>1</td>
<td>3.50E+03</td>
<td>1.78E+04</td>
<td>4.29E+05</td>
<td>1.42E+08</td>
<td>2.43E+08</td>
<td>5.67E+08</td>
</tr>
<tr>
<td>AcVP39:HA_FLAG</td>
<td>1</td>
<td>9.54E+02</td>
<td>4.99E+03</td>
<td>1.40E+05</td>
<td>3.33E+07</td>
<td>3.74E+08</td>
<td>1.36E+09</td>
</tr>
<tr>
<td>AcHA:GP64_FLAG</td>
<td>1</td>
<td>1.43E+03</td>
<td>2.68E+03</td>
<td>6.83E+04</td>
<td>1.56E+07</td>
<td>7.45E+07</td>
<td>1.20E+09</td>
</tr>
<tr>
<td>AcHA_FLAG</td>
<td>1</td>
<td>8.66E+03</td>
<td>4.49E+04</td>
<td>2.70E+05</td>
<td>1.61E+07</td>
<td>3.90E+07</td>
<td>3.89E+08</td>
</tr>
<tr>
<td>AcΔCC</td>
<td>1</td>
<td>1.55E+03</td>
<td>2.94E+04</td>
<td>2.70E+05</td>
<td>3.84E+07</td>
<td>6.35E+08</td>
<td>1.02E+09</td>
</tr>
</tbody>
</table>
Table 6.3 Single-Factor ANOVA: Microsoft Excel Output for Statistical analysis of variance between growth of recombinant HA-expressing viruses

Anova: Two-Factor Without Replication

<table>
<thead>
<tr>
<th>SUMMARY</th>
<th>Count</th>
<th>Sum</th>
<th>Average</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Row 1</td>
<td>6</td>
<td>5E+08</td>
<td>83258005</td>
<td>9.69E+15</td>
</tr>
<tr>
<td>Row 2</td>
<td>6</td>
<td>2.07E+09</td>
<td>3.46E+08</td>
<td>1.76E+17</td>
</tr>
<tr>
<td>Row 3</td>
<td>6</td>
<td>1.76E+09</td>
<td>2.94E+08</td>
<td>2.94E+17</td>
</tr>
<tr>
<td>Row 4</td>
<td>6</td>
<td>1.77E+09</td>
<td>2.96E+08</td>
<td>2.94E+17</td>
</tr>
<tr>
<td>Row 5</td>
<td>6</td>
<td>2.49E+09</td>
<td>4.15E+08</td>
<td>1E+18</td>
</tr>
<tr>
<td>Row 6</td>
<td>6</td>
<td>4.1E+08</td>
<td>68265033</td>
<td>1.15E+16</td>
</tr>
<tr>
<td>Row 7</td>
<td>6</td>
<td>6.3E+08</td>
<td>1.05E+08</td>
<td>5.22E+16</td>
</tr>
<tr>
<td>Row 8</td>
<td>6</td>
<td>2.58E+08</td>
<td>43052520</td>
<td>6.7E+15</td>
</tr>
<tr>
<td>Row 9</td>
<td>6</td>
<td>2.73E+09</td>
<td>4.55E+08</td>
<td>4.88E+17</td>
</tr>
<tr>
<td>Row 10</td>
<td>6</td>
<td>6.67E+08</td>
<td>1.11E+08</td>
<td>3.65E+16</td>
</tr>
</tbody>
</table>

| Column 1 | 10    | 32163 | 3216.3 | 24438029 |
| Column 2 | 10    | 199447 | 19944.7 | 3.42E+08 |
| Column 3 | 10    | 2354410 | 235441  | 3.02E+10 |
| Column 4 | 10    | 1.16E+09 | 1.16E+08 | 6.11E+16 |
| Column 5 | 10    | 2.73E+09 | 2.73E+08 | 1.04E+17 |
| Column 6 | 10    | 9.4E+09 | 9.4E+08 | 5.45E+17 |

ANOVA

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rows</td>
<td>1.31E+18</td>
<td>9</td>
<td>1.45E+17</td>
<td>1.285634</td>
<td>0.271092</td>
<td>2.095755</td>
</tr>
<tr>
<td>Columns</td>
<td>6.77E+18</td>
<td>5</td>
<td>1.35E+18</td>
<td>11.97277</td>
<td>2.13E-07</td>
<td>2.422085</td>
</tr>
<tr>
<td>Error</td>
<td>5.09E+18</td>
<td>45</td>
<td>1.13E+17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1.32E+19</td>
<td>59</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix B

Sample Calculation of Molecular Weight using Linear Regression

Figure A1: Standard Curve generated from plotting the \( \log_{10} \) of the known molecular weight of several proteins from the Frogga Bio Protein Ladder employed in these experiments against the distance migrated (cm). The equation of the linear trendline is displayed on the graph.

Using the equation of the line from the standard curve:

Measured distance band 1 migrated: 1.8cm

\[
Y = -0.3212x + 2.3792
\]

\[
Y = -0.3212(1.8) + 2.3792
\]

\[
Y = 1.801
\]

\[
MW = 10^{1.801}
\]

\[
MW = 63.24\text{kDa}
\]
Sample Calculation of Protein from a BSA Standard Curve

Figure A2: Graph of $A_{595}$ readings of known concentrations of BSA. The equation of the linear trendline is displayed on the graph.

Sample Calculation

Protein of interest $A_{595}= 1.94$

$y=0.0011x + 0.5667$

$1.94 = 0.0011x + 0.5667$

$1.94 - 0.5667/0.0011= x$

$1248 \mu g/ml$
Appendix D

Raw data from NA assays

Table A4. Raw A\textsubscript{560} readings from three individual trials using Neuraminidase assay kit. Mean A\textsubscript{560} readings and standard deviations are also displayed in the table.

<table>
<thead>
<tr>
<th>Trial</th>
<th>AcNA\textsubscript{HA} 96 h.p.i.</th>
<th>CHIA(ASD)\textsubscript{FLAG}</th>
<th>Empty Well</th>
<th>H2O2</th>
<th>NA Pos Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.193206</td>
<td>0.165113</td>
<td>0.090045</td>
<td>0.577893</td>
<td>0.329497</td>
</tr>
<tr>
<td>2</td>
<td>1.010414</td>
<td>0.129975</td>
<td>0.0886</td>
<td>0.581425</td>
<td>0.314984</td>
</tr>
<tr>
<td>3</td>
<td>0.924617</td>
<td>0.101834</td>
<td>0.087606</td>
<td>0.586387</td>
<td>0.307329</td>
</tr>
<tr>
<td>Average</td>
<td>1.042746</td>
<td>0.132307</td>
<td>0.08875</td>
<td>0.581902</td>
<td>0.31727</td>
</tr>
<tr>
<td>StDEV</td>
<td>0.137183</td>
<td>0.031704</td>
<td>0.001226</td>
<td>0.004267</td>
<td>0.01126</td>
</tr>
</tbody>
</table>