DELETION AND FUNCTIONAL ANALYSIS OF AC146 OF THE BACULOVIRUS ` AUTOGRAPHA CALIFORNICA NUCLEOPOLYHEDROVIRUS

by

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ABSTRACT

Autographa californica Multiple Nucleopolyhedrovirus (AcMNPV) *ac146* is a highly conserved gene in the alpha- and betabaculovirus genera that has an unknown function. To characterize *ac146* Northern blot analysis and transcriptional mapping confirmed the prediction that *ac146* is transcribed at late times post infection as a 1.2 Kb mRNA that is detected from 12 hpi through to 48 hpi. To determine the role of *ac146* in the baculovirus life cycle, AcMNPV bacmids were used to generate a series of four increasingly larger *ac146* deletion viruses (AcBAC$^{ac146\text{KO1 - 4}}$) by recombination in *Escherichia coli*. Transfection and plaque assays were completed to visualize the movement of *ac146* deletion and repair viruses under fluorescence and light microscopy. The results showed that all the *ac146* deletions produced a single cell phenotype indicating that no infectious budded virus (BV) was produced. Lack of BV production was confirmed by titrating the virus utilizing both qPCR and $TCID_{50}$. Within cells transfected by AcBAC$^{ac146\text{KO1 - 4}}$ the infection preceded to late times post-infection as evidenced by the development of apparently normal occlusion bodies (OB). AC146 was detected at 18 hpi through to 96 hpi and located in both nuclear and cytoplasmic fractions at 24 and 48 hpi. Purification of BV and occlusion derived virus (ODV) revealed that AC146 is associated with both forms of the virus. AC146 was located in the nucleocapsid fractionation of BV but not in the envelope fraction. Therefore in conclusion, this study has shown that *ac146* is a late gene that is essential for the virus life cycle and is required for the production of BV.

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LIST OF ABBREVIATIONS

aa	amino acid
Ab	antibody
AcMNPV	*Autographa californica* multiple nucleopolyhedrovirus
BmNPV	*Bombyx mori* nucleopolyhedrovirus
BLAST	basic local alignment search tool
bp	base pair
BV	budded virus
CAT	chloramphenicol acetyltransferase
CIP	calf intestinal phosphatase
C-terminal	carboxy-terminal
DE	delayed early
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphatases
ds	double stranded
Fig.	Figure
g	gram
GP64	glycoprotein 64
hpi	hours post infection
hpt	hours post transfection
gfp	green fluorescent protein
GV	immediate early
F protein	fusion protein
ICTV	International Committee on Taxonomy of Viruses
kb	kilobase
kDa	kilodalton
L	late
Ld_{50}	lethal dose to kill 50%
LdMNPV	*Lymantria dispar* multiple nucleopolyhedrovirus
Lef	late expression factor
µ	micro
m	milli
M	molar in the context of concentration
MAb	monoclonal antibody
ml	millilitre
mM	millimolar
min	minute(s)
mRNA	messenger RNA
MOI	multiplicity of infection
MSC	multiple cloning site
NCBI	National Center of Biotechnology Information
nt	nucleotides
N-terminal	amino-terminal
NPV	Nucleopolyhedrovirus
OB	occlusion body

ODV	occlusion derived virus
OpMNPV	*Orgyia pseudosugata* multiple nucleopolyhedrovirus
ORF	open reading frame
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PIF	per os infectivity
PVDF	polyvinylidene difluoride
qPCR	quantitative PCR
RNA	ribonucleic acid(s)
SDS	sodium deodecyl sulfate
Sf	*Spodoptera frugiperda*
ss	single stranded
TBS	Tris buffered saline
$TCID_{50}$	tissue culture infectious dose 50%
UAS	upstream activating sequence
UTR	untranslated region
UV	ultraviolet
VL	very late
VLF	very late expression factor
VP	viral protein
VLF	very late factor
WT	wild type

DEDICATION

I would like to dedicate this thesis to my family.

First to my husband, Carl, thank you for being so supportive throughout the past two years. I couldn't have done this without your support!! It is your turn now, and I promise to be as patient with you as you were with me.

To my mother and father: Thank you for teaching me what hard work and dedication are and where those attributes will take you in life. To my brothers Joshua and Jonathan: Josh, thank you for being so wise and always having such comforting and sound advice. A big thank you to Jonathan for always making me laugh.

To my Grammy and late Grampy Chamberlain, I would like to express my gratitude for being there for me when I needed you and for taking care of me when I wanted to be home. Grammy Chamberlain thank you for always being "a little you" and to Grammy Dickison for being an amazing person. I would also like to thank Grampy Dickison for setting an example of what it is to work hard.

Finally, to my son, Caleb, thank you for being angel and not punishing me for being so distracted for the first seven months of your life. I promise I will make it up to you.

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Finally, I would like to thank Dr. Gary Blissard for the use of the AcMNPV GP64 knockout virus.

1 Introduction

Baculoviruses are a family of arthropod-specific viruses mainly infecting the insect orders Lepidoptera, Hymenoptra and Diptera. This family of double-stranded (ds) DNA viruses has over 600 described viruses in the literature encompassing one of the most diverse and largest groups of viruses (Herniou and Jehle, 2007; Herniou et al., 2003). Baculovirus infections were first alluded to over 5000 years ago in reference to the silk industry in China. The first known baculovirus was observed infecting the silkworm as it was a major hindrance for silk production, of cultural importance in both the Chinese and Japanese cultures (Friesen and Miller, 1985; Rohrmann, 2008b). Baculovirus infections were identified by the release of their characteristic occlusion body (OB) form from an infected silkworm. The virus was named polyhedrosis disease because of the polyhedral appearance of the occlusion bodies.

A great deal of investigation has gone into the study of baculoviruses as they have an innate ability to control insect populations and do so in a host-specific manner. This attribute makes them a logical choice as an environmentally sustainable biological control agent (van Oers and Vlak, 2007). Baculoviruses have also been used extensively in the biotechnology industry as gene expression vectors and in addition, are currently being tested as gene therapy vectors (Condreay and Kost, 2007).

The majority of sequenced baculovirus genomes are derived from viruses that infect economically important insect pest species that are responsible for agricultural crop damage. To date, 50 baculovirus genomes have been sequenced containing from 80 to over 160 predicted genes (NCBI genome database). The genomes sequenced are from baculoviruses that infect Lepidoptera (46), Hymenoptera (3) and a single virus from Diptera. Genomic comparisons have revealed 31 core genes occurring in all of the 50 sequenced genomes

(Fang et al., 2009). Lepidopteran baculoviruses share another 33 genes in addition to the core genes, of which 16 have yet to be characterized (van Oers and Vlak, 2007).

The virus that will be used in this study will be *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) which is the type species for the alphabaculovirus genus. The objective of this research was to characterize and determine the function of *ac146*, a highly conserved gene in the AcMNPV genome. Highly conserved genes of baculoviruses have been shown to play critical roles in the baculovirus life cycle. Therefore *ac146* was expected to play a key role in the viral life cycle; potentially in DNA processing, occlusion body formation or budded virus production.

1.1 Baculoviridae

Baculoviruses contain large, dsDNA genomes ranging from approximately 80 to approximately 180 kbp in size. The super-coiled, circular dsDNA is packaged into rod shaped nucleocapsids. Baculoviruses have biphasic life cycles which enclose the nucleocapsids into one of two virion phenotypes; the budded virus (BV) or the occlusion derived virus (ODV) (Fig. 1). Occlusion bodies enclose ODVs in a protein (polyhedrin/granulin) matrix that is responsible for persistence and protection of virions after release into the environment (Olszewski and Miller, 1997a).

1.2 Phylogeny

Baculovirus phylogeny has made major strides with innovative molecular technology, particularly with the advance in genome sequencing abilities. Currently four genera; alpha -, beta -, gamma - and deltabaculoviruses are recognized based on phylogenetic evidence,

genome composition, pathological traits and morphology. A new naming system was recently accepted by the International Committee on Taxonomy of Viruses (ICTV) (Fig. 2). Alphabaculoviruses and betabaculovirus encompass the lepidopteran baculoviruses, whereas the gamma- and deltabaculovirus genera consist of the hymenpteran-specific baculoviruses and the dipteran baculoviruses, respectively (Herniou and Jehle, 2007; Jehle et al., 2006). OBs can be ovoid and contain a single virion (betabaculoviruses) or much larger, polyhedral in shape, and contain many virions. Virions of alphabaculoviruses may contain multiple (M) or a single (S) nucleocapsid per ODV whereas delta- and gammabaculoviruses normally contain single nucleocapsids per ODV (Herniou, 2007).

Alphabaculoviruses can be divided into two subgroups based upon the use of different envelope fusion proteins for secondary infection. The two budded virus envelope fusion proteins are GP64 used by the group I alphabaculoviruses and a furin-cleaved (F) protein which is utilized by the group II alphabaculoviruses (Blissard and Rohrmann, 1991; Herniou et al., 2003; Pearson and Rohrmann, 2002; Rohrmann, 1992; Slack and Arif, 2007).

1.3 Baculovirus gene expression and genome structure

Baculoviruses genes are expressed in a cascade of regulatory events that rely on the genes in the preceding class for the proceeding class of genes to be expressed (Friesen and Miller, 1985). The class of the gene does not determine its location on the genome; genes are disbursed throughout the genome regardless of their temporal expression (van Oers and Vlak, 2007). There are four classes of genes that are classified according to their temporal expression during the infection cycle; immediate early (IE), delayed early (DE), late (L), or very late (VL) genes (Guarino and Summers, 1987). IE genes are transcribed using host

factors and do not require viral genes for expression. Delayed early genes require viral gene products for their transcription such as *ie1*, a regulatory gene. Late genes are transcribed after or during the onset of viral DNA synthesis and initiate from the late-gene promoter motif (DTAAG) using a virally encoded RNA polymerase (Blissard and Rohrmann, 1990). Very late (VL) genes are hyper-expressed genes that are transcribed after viral DNA synthesis and include the genes *polyhedrin* and *p10*. VL genes in addition to the late RNA polymerase require very late factor – 1 (VLF-1) for their hyperexpressed levels of late gene expression (Mistretta and Guarino, 2005; van Oers and Vlak, 2007).

AcMNPV, which is the virus used in this study, infects lepidopterans and has a 134 kbp genome with 154 predicted open reading frames (ORFs) (Ayres et al., 1994). AcMNPV has a very broad host range, which is an abnormal trait of baculoviruses, infecting 43 species in 11 different families. This broad host range indicates that AcMNPV has evolved an effective strategy for overcoming host defenses (Volkman, 2007).

1.4 Baculovirus life cycle

Baculoviruses utilize a biphasic life cycle to optimize infection of their host. Initiation of infection occurs when OBs are ingested by a susceptible host. Susceptible hosts are always juvenile or larval arthropods (Slack and Arif, 2007; Volkman, 2007) which ingest OBs that were deposited on foliage by an infected host (Fig. 2). After oral or "per os" infectivity of OBs, they are passed through the foregut and enter the midgut. The midgut of the host is an alkaline environment that causes dissolution of the protein matrix surrounding the ODVs. Upon release of the ODV they attach and pass through the peritrophic membrane at the

apical ends of the microvilli to enter the midgut tissues. The entry of the ODV is aided by multiple proteins called enhancins that are located on the ODV envelope.

Once inside the midgut cells the nucleocapsids make there way through the cytoplasm to the nuclear envelope where virions are unpackaged and the viral genome is released into the nucleus. Within the nucleus a virogenic stroma is formed where viral DNA replication occurs and nucleocapsids are assembled that will either become packaged into BV or ODV. Midgut cells are often sloughed from the insect as a defense mechanism; therefore, rapid assembly and egress of BV must occur for systemic infection of the organism to be successful. The infection beyond the initial midgut infection is termed secondary infection (Slack and Arif, 2007; Volkman, 2007). It has been recognized that occasionally baculovirus nucleocapsids bypass the midgut cell nucleus and replication process and bud directly from the midgut cell to more rapidly initiate a systemic infection (Slack and Arif, 2007). The tracheal system is also commonly used as a route of secondary infection.

Late in infection there appears to be a shift in the packaging of nucleocapsids. Instead of nucleocapsids becoming BV the nucleocapsids are retained in the nucleus and become ODVs. The mechanism that regulates the change in production from BV to ODV has yet to be uncovered. Rather than obtaining their nucleocapsid envelope by budding from the cell, the envelope of nucleocapsids destined to become ODVs gain a lipid bilayer envelope in the nucleus that resembles the inner nuclear membrane (Braunagel and Summers, 2007). At very late times post infection the virus focuses production on the hyper-expressed very late genes, *polyhedrin* and *p10* to form the protective OB (van Oers and Vlak, 2007). Once the cell is filled with many occlusion bodies the cell lyses. Due to the expression of proteases

and chitinases there is a "liquefying" of the host which releases OBs for another susceptible host to ingest (Fig. 1) (Rohrmann, 2008b; Slack and Arif, 2007; Volkman, 2007).

1.5 Viral entry

The two virion forms of baculovirus biphasic life cycle are very different; therefore, alternate envelope fusion mechanisms are necessary. The primary infection of ODV with the host midgut is not a well understood mechanism appearing to be much more difficult than the well studied entry of BV during secondary infection. This section of the literature review will take a closer look at primary and secondary infection along with the major proteins known to play key roles in this process.

1.5.1 Primary infection

Primary infection occurs in the midgut of susceptible hosts. The host ingests OBs that were usually deposited on plant tissue by a previously infected host. The OBs travel with the ingested tissue through the foregut and into the midgut lumen. The midgut lumens have an alkaline environment that dissolves the polyhedrin/granulin matrix which in turn releases the ODV. The ODV attach and fuse with the midgut columnar epithelial cells. The exact mechanism for entry is not known but studies have revealed that ODV attach to the tips of the apical microvilli of the columnar cells. Two types of proteins have been suggested as being involved in ODV access to the midgut columnar epithelial cells, enhancins and the 11k proteins (Rohrmann, 2008a; Volkman, 2007). Enhancin proteins which are only found in some baculoviruses, such as the betabaculoviruses, are metalloproteases that digest mucin. The digestion of mucin, a component found in the peritrophic membrane, allows the ODV to

contact the epithelial surface where attachment occurs. The 11K proteins are members of a gene family that contain a chitin-binding domain. These genes are more prevalent in baculoviruses than the enhancin proteins. Chitin, like mucin, is a component of the peritrophic membrane (Rohrmann, 2008a; Volkman, 2007).

The PIF (per os infectivity) proteins, p74, PIF 1, PIF2, PIF3 and PIF 4 are also thought to affect binding of ODV to the epithelial cells of the midgut. PIF genes are involved in specific binding to midgut cells and are critical in determining the host range of the virus. These proteins are viral attachment proteins that significantly affect oral infection rates when deleted from the genome (Fang et al., 2009; Kuzio et al., 1989; Ohkawa et al., 2005; Pijlman et al., 2003).

ODV-E66 has been identified as a hyaluronan lyase (Vigdorovich et al., 2007) which has the ability to digest a component of the extracellular matrix polysaccharide, hyaluronan. This activity would aid in the virus' ability to gain access to the midgut epithelial cells in an expedient manner. It is proposed that hyaluronan lyases are involved in the fusion of the virus with the midgut cells (Rohrmann, 2008a). It was also postulated by Vigdorovich et al. (2007) that ODV-E66 was involved in the exit from the cell after infection has occurred. This hypothesis seems unwarranted as the virions are enclosed in a protein matrix when infection is complete and lysis of the cell occurs; therefore, the ODV-E66 protein would not have access to cellular components at this time. It is more likely that the protein would be involved in ODV entry to the epithelial midgut cells

Much of the ODV viral entry is speculative at this time. Identification and functional analysis of additional proteins that are associated with ODV are bringing us closer to a clearer picture of the exact process of primary infection.

1.5.2 Secondary infection

Secondary infection is accomplished using the BV form of the baculovirus which causes systemic infection of the host. Nucleocapsids produced in the nucleus travel to the plasma membrane where they acquire their envelope which contains the fusion protein by budding through the plasma membrane. The fusion protein for the group I alphabaculoviruses is GP64, whilst the fusion protein for the remainder of baculoviruses is the F protein. Both fusion proteins have similar functions but are structurally different from one another (Lung et al., 2003).

Budding of the virus from the midgut cells can occur in two ways; directly by ODV nucleocapsids passing through the midgut cells and after the synthesis and assembly of new nucleocapsids. Systemic infection occurs when BVs bud out of the midgut cells, through the basement membrane and penetrates the basal lamina, subsequently infecting the tracheal system. Once BVs enter the tracheal system this leads them to the hemocoel where all cells can be accessed leading to systemic infection (Rohrmann, 2008a; Slack and Arif, 2007).

1.6 Structural proteins of the nucleocapsid, ODV envelope and BV envelope

This section of the literature review will focus on the proteins involved in the structure of the nucleocapsids, ODV envelope and BV envelope.

1.6.1 Nucleocapsid proteins

Nucleocapsids of ODV and BV are believed to contain the same proteins and structure. Nucleocapsids have an apical cap at one end and a basal end that appears “claw like” in form at the other. Nucleocapsid size, which is dependent upon the variable size of the genome (80

kb-180 kb), ranges from 40-70 nm in diameter and 250-400 nm in length. Determining proteins that are essential for the structural integrity of the nucleocapsid can prove difficult as associated proteins can become enclosed within the capsid but are not essential for nucleocapsid formation. The use of bacmid deletion experiments has resolved this problem somewhat from the previously used methods of antibody detection and mass spectrometry, although new questions arise with the utilization of bacmid technology (Rohrmann, 2008b; Slack and Arif, 2007). Although issues identifying proteins of the nucleocapsid structure have occurred, we will now take a closer look at some of the key genes that have been identified as part of the nucleocapsid and play major structural roles. This review of nucleocapsid proteins will focus on the overall structure of nucleocapsids and the major proteins associated with the nucleocapsid.

Nucleocapsids are mainly composed of one protein, VP39 (viral structural protein 39), that forms a ring-like structure around the nucleoprotein core (Fig. 3) (Slack and Arif, 2007; Thiem and Miller, 1989). A protein that has been reported to assist in the arrangement of VP39 in the nucleocapsid is VP1054. This protein is also essential as temperature sensitive mutants of VP1054 were unable to produce BV (Olszewski and Miller, 1997b). Also identified interacting with VP39 in the nucleocapsid structure are VP80 (Lu and Carstens, 1992), P6.9 and VFL-1. VFL-1 is reported to have an alternate role involving the transcription of very late genes as previously described (Vanarsdall et al., 2006) and P6.9 has been identified as a DNA binding protein (Wilson et al., 1987).

At the basal structure of the nucleocapsid three proteins have been identified interacting with one another; PP78/83, BV/ODV E27, and BV/ODV C42. PP78/83 is proposed to translocate nucleocapsids to the nucleus after infection (Lanier and Volkman, 1998). ODV-

EC27, which has also been shown to associate with the ODV envelope, is a multifunctional viral cyclin which is thought to arrest cell cycle (Belyavskyi et al., 1998). BV/ODV-C42 is predicted to also play a role in regulating cellular functions during infection (Braunagel et al., 2001).

EXON0 (AC141) is a nucleocapsid protein that was determined to be necessary for efficient egress of nucleocapsids from the nucleus (Fang et al., 2007). *Exon0* is transcribed both early and late during infection. The *exon0* early promoter region transcribes an mRNA of 114 bp that is spliced onto *ie1* to generate the only known spliced gene in the AcMNPV genome (Dai et al., 2004). The late promoter transcribes the nucleocapsid protein EXON0 that interacts with microtubules for efficient egress of nucleocapsids from the nucleus (Fang et al., 2009).

Two proteins have recently been added to the growing list of nucleocapsid-associated proteins. These genes identified in the AcMNPV genome are *ac142* and *ac143*. AC142 appears to be a multifunctional protein (Braunagel et al., 1996b) including being required as an essential nucleocapsid structural protein (McCarthy and Theilmann, 2008;Vanarsdall et al., 2007). AC142 may also interact with the ODV envelope protein AC143 resulting in envelopment of nucleocapsids in the nucleus prior to occlusion (McCarthy and Theilmann, 2008).

The protein GP41 is the only known O-glycosylated protein in the baculovirus genome and has been identified as an essential component of nucleocapsid formation and aids in the egress of nucleocapsids from the nucleus (Whitford and Faulkner, 1993). GP41 is said to be located between the nucleocapsid and the envelope, in an area called the tegument (Slack and Arif, 2007).

1.6.2 ODV envelope proteins

In association with the ODV envelope there are many viral encoded proteins. Of the proteins associated with the ODV envelope there are two types; structural proteins involved in the occlusion of virions and the PIF genes that interact with the midgut during oral infection. The ODV envelope is acquired in the nucleus, the exact origin of the envelope is unknown but it is hypothesized that the lipid bilayer envelope (Slack and Arif, 2007) originates from mircrovesicles budding from the inner nuclear membrane (Braunagel and Summers, 2007). To date there have been 15 ODV envelope proteins identified. Eight of those identified as structural proteins and 7 reported as proteins involved in oral infectivity P74 (PIF0), PIF 1, PIF 2, PIF 3, PIF 4, 11K proteins (ac145/ac150), and in some enhancins (Fig. 4) (Braunagel and Summers, 2007; Fang et al., 2009; McCarthy and Theilmann, 2008; Rohrmann, 2008b; Slack and Arif, 2007).

A protein combination thought to assist nucleocapsid envelopment to form ODV is the FP25K complex. FP25K interacts directly with ODV-E66 and ODV-E26 and indirectly with ODV-E25 and VP39. ODV-E66 and ODV-E25 have been shown to interact with one another at the C-terminal ends on the inner side of the ODV envelope. Further interaction of ODV-E66 and ODV-E25 with the major nucleocapsid protein VP39 has been illustrated suggesting that ODV- E66 and ODV-E25 are involved in ODV envelopment (Braunagel et al., 1999).

The interaction of the PIF genes with the midgut of the host are not only responsible for the initiation of horizontal transmission but they may also determine the host range of the virus (Braunagel and Summers, 2007). Many of the genes identified as ODV envelope proteins have N-terminal transmembrane sequences and signal sequences that target the gene

to the nucleus (Rohrmann, 2008b). All the identified PIF, are recognized as belonging to the 31 core genes that are found in all baculoviruses and their conservation indicates their importance in the viral life cycle.

1.6.3 BV envelope proteins

In comparison to the complex nature of the ODV envelope, the BV envelope appears to be simple in structure. The BV envelope contains the fusion protein GP64 or F protein which appears to be essential for BV infectivity as opposed to the 7 PIF proteins destined to become a component of the ODV envelope. The envelope of BV is obtained when virions egress from the cell through the plasma membrane. Approximately 16% of nucleocapsids that are produced ultimately become BV (Braunagel and Summers, 2007). These nucleocapsids egress from the nucleus to where the fusion proteins are located. The fusion proteins have already been integrated into the plasma membrane by means of trafficking through the ER. BV envelope proteins are N-glycosylated, sorted in the endoplasmic reticulum and then transported to the plasma membrane (Rohrmann, 2008b; Slack and Arif, 2007). The envelope proteins of BV appear as peplomers emerging from the bulbous end of the BV or are found over the entire BV envelope.

1.7 AcMNPV *ac146*

The ORFs of the AcMNPV genome are densely packed with many genes having overlapping regulatory sequences. The gene *ac146* is conserved in all alpha- and betabaculoviruses and its ORF is located within the promoter sequences of the essential gene *ie1* (Theilmann and Stewart, 1991). *Ie1* and its spliced counterpart *ie0* are the primary

AcMNPV transregulatory genes. IE0 and IE1 proteins are essential for the baculovirus life cycle and transactivate transcription of early and late genes. In addition, IE0 and IE1 play a critical role in initiating baculovirus DNA replication and may act as an origin binding protein (Friesen, 1997). A previous study by Stewart *et al.* (2005) analyzed the function of IE1 by generating a knockout (KO) mutant of AcMNPV that removed the entire *ie1* ORF. The results of this study showed that the virus was completely inactive indicating that *ie1* is essential for wild type virus replication. A review of the experimental design of this study identified that during the construction of the *ie1* KO the predicted promoter region for the highly conserved *ac146* was also deleted. Therefore it is possible that the viral phenotype observed by deleting the *ie1* ORF could be due to the result of a double knockout involving both *ie1* and *ac146*.

There are no specific publications concerning *ac146* but a previous study on the *ie1* gene region (Guarino and Summers, 1987) suggested that *ac146* may be transcribed late in the viral life cycle as a 1.2 kb transcript. The AcMNPV *ac146* ORF encodes a predicted 201 amino acid protein but no further studies have been performed on this gene. In addition to the possible role in *ie1* expression and since *ac146* is one of the most highly conserved genes sequenced to date it is possible that it serves a key role in the viral life cycle. Determining the role of *ac146* in the AcMNPV life cycle will be necessary for dissecting the molecular mechanisms of baculovirus replication.

1.8 Study hypothesis

Ac146 is a highly conserved gene in the baculovirus genome and therefore is essential for the AcMNPV life cycle.

1.9 Study objectives

The objective of this study was to characterize and determine the function *ac146*, a highly conserved gene in the AcMNPV genome. The objectives were met by characterizing AC146 through transcriptional analysis and determining its function by constructing recombinant baculoviruses with mutations at the *ac146* loci and analyzing the replication of the AC146 mutants.

1.10 Significance of the study

Determining the molecular mechanisms by which baculoviruses infect and kill cells is essential for understanding viral virulence. It is therefore necessary to understand the function of all the genes within the viral genome especially those that are highly conserved. Elucidating the roles of all the genes within the virus also aids in the development of future viral modifications for use as a biological control agents or for use in gene expression systems. *Ac146* is conserved in all lepidopteran baculoviruses and therefore is likely to play a significant role in the infection process. This will be the first study to characterize and functionally analyze *ac146*. The proposed study will further our knowledge of the genes present in the lepidopteran baculoviruses and also provide fundamental knowledge about eukaryotic viral gene expression and replication. The highly conserved genes of baculoviruses have been shown to play critical roles in the baculovirus life cycle. Therefore *ac146* is expected to play a key role in the viral life cycle; potentially in DNA processing, occlusion body formation or budded virus production.

Figures

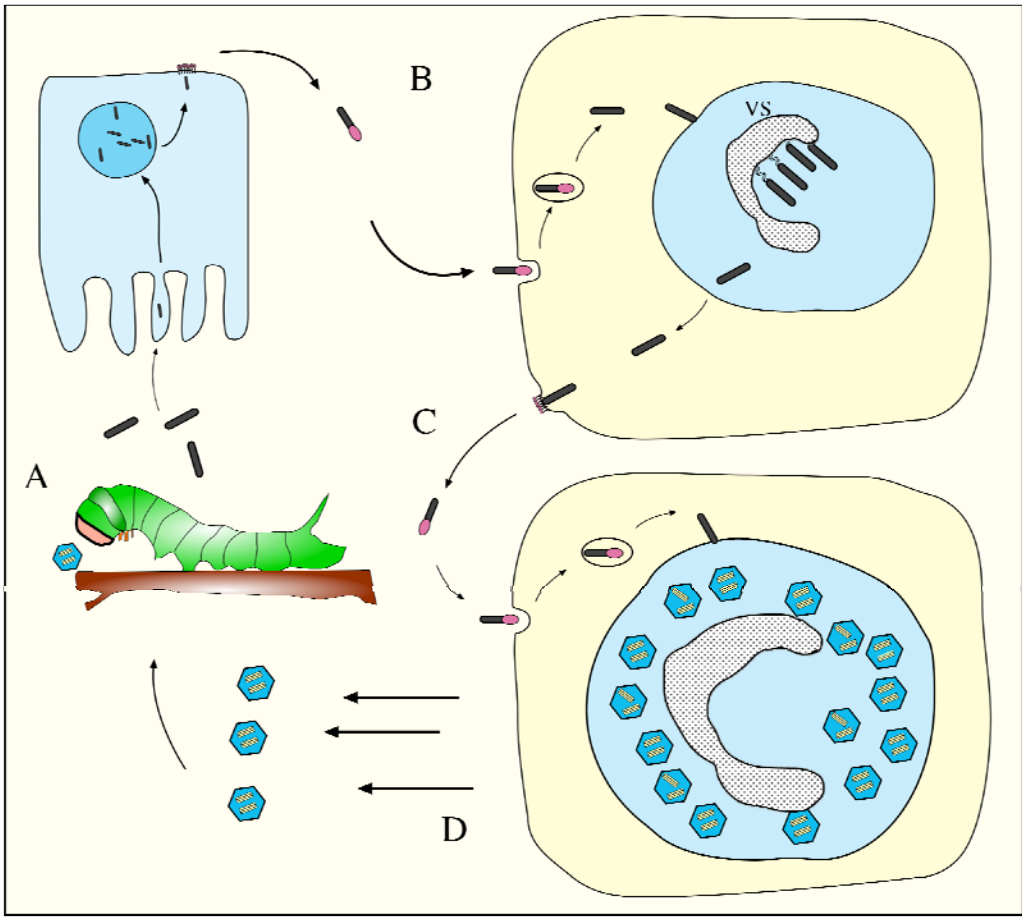

Fig. 1. A schematic depicting the life cycle of a baculovirus infection of an insect cell. (A)The infection begins as an occlusion body entering its host via ingestion and travels through to the midgut where the alkaline environment of the midgut dissolves the polyhedral coating surrounding the ODV. The ODV are released and infect the epithelial cells. (B) Virions bud from the cell inducing secondary infection. (C) Secondary infection occurs until the virus has infected the majority of cells. (D) Late times infection ODV are produced causing the cells to lyse. The virogenic stroma is indicated as VS within the nucleus (Rohrmann, 2008a). Republished with permission from Dr. George Rohrmann.

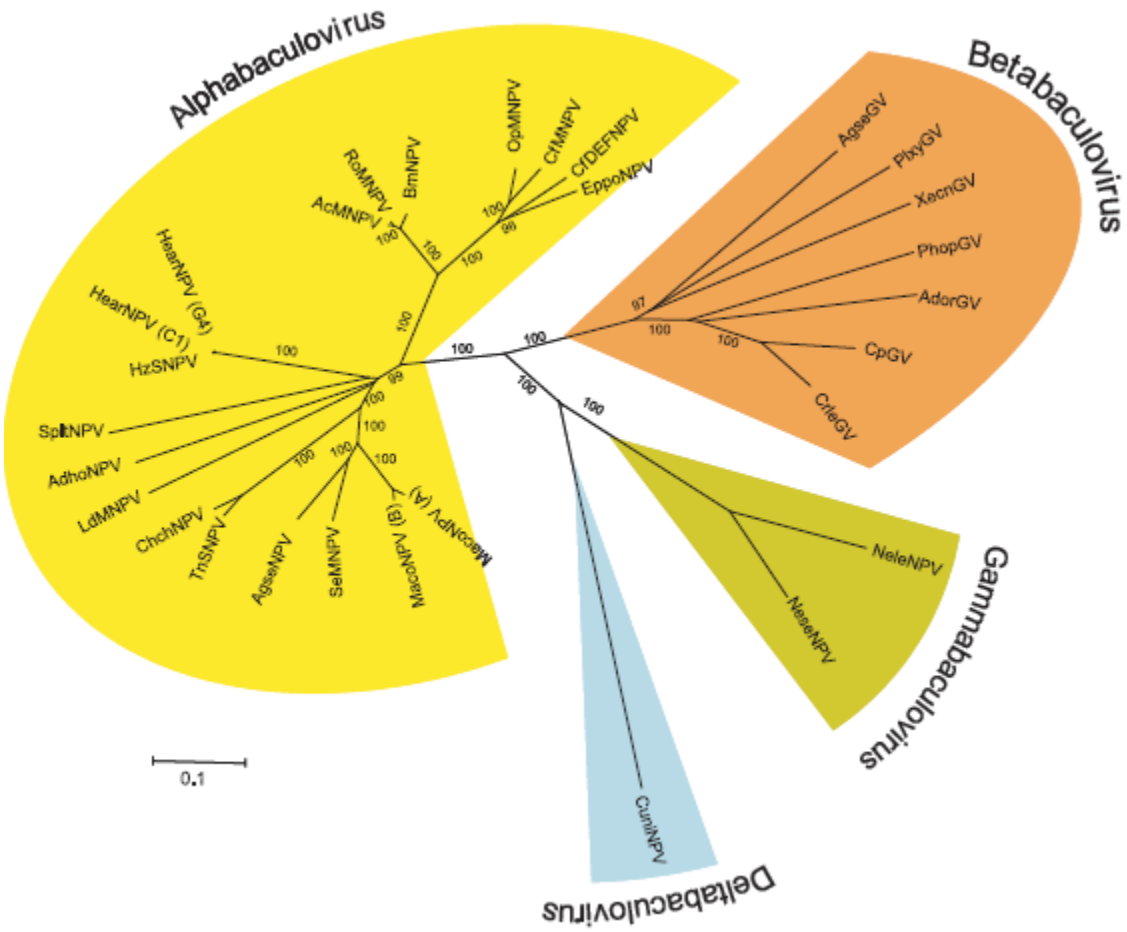

Fig. 2. A neighbor-joining tree representing the relatedness of baculoviruses using 29 core genes was taken from analysis done by Jehle *et al*. (2006). The baculovirus genera are divided into alpha -, beta - , delta – and gamma – baculoviruses which are labeled in the figure. Republished with permission.

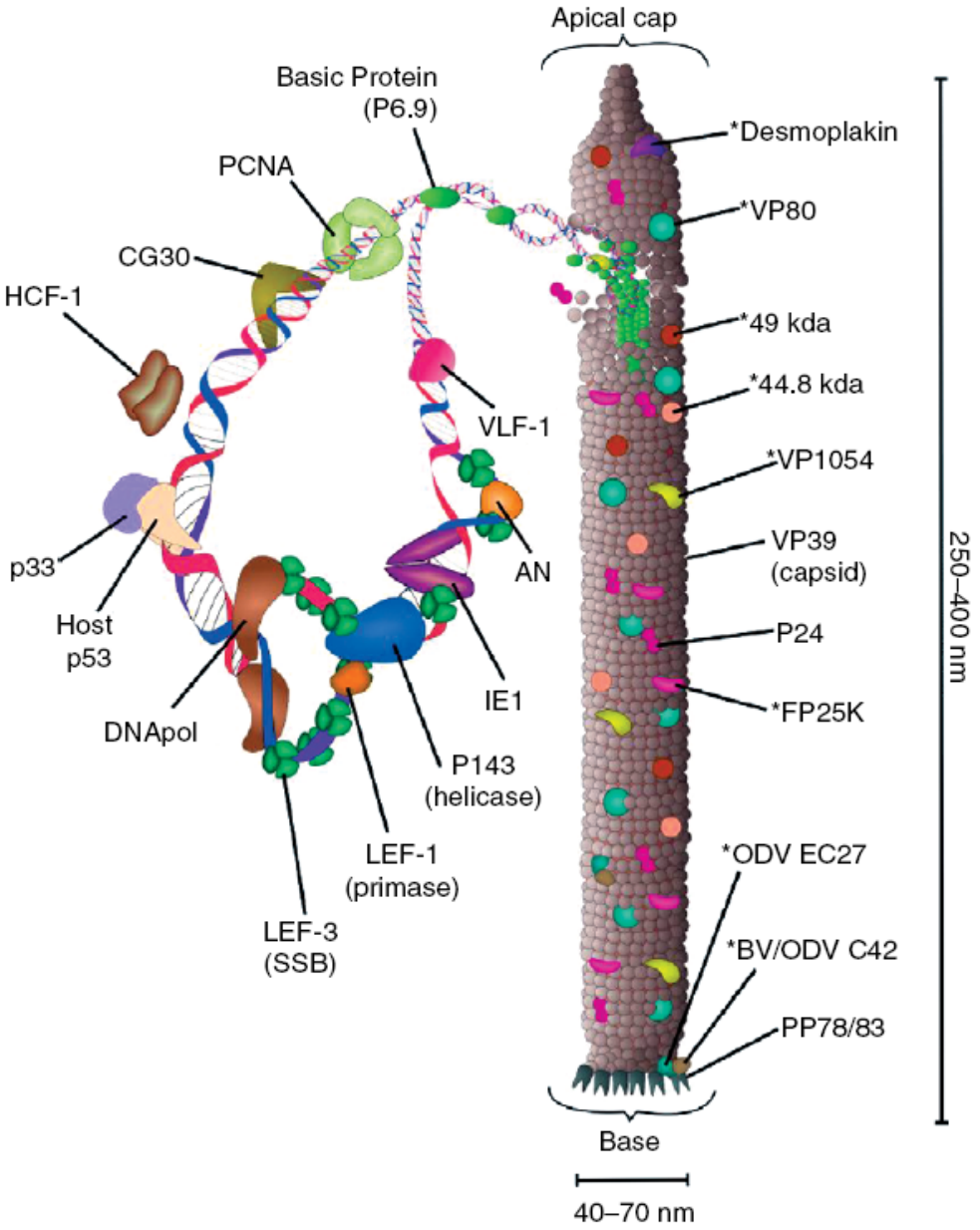

Fig. 3. Baculovirus nucleocapsid and proteins that have been identified as associated with the nucleocapsid (Slack and Arif, 2007). Nucleocapsid-associated proteins recently been identified are absent from this diagram. These proteins are *exon0*, *ac142* and *ac143* (*odv-e18*). Republished with permission.

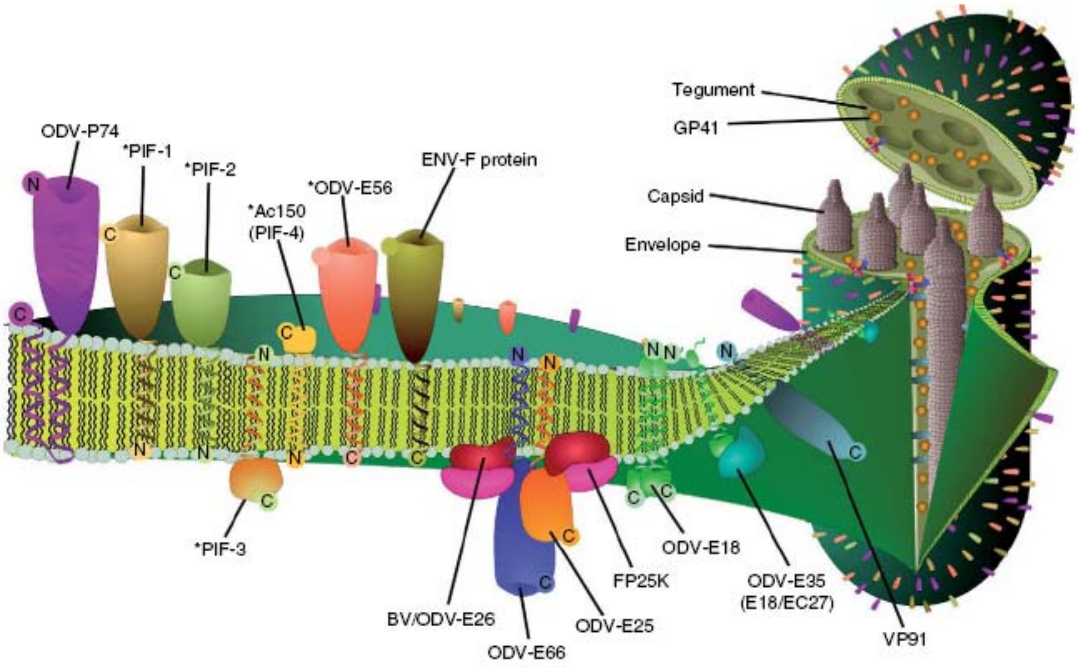

Fig. 4. Proteins interacting with the ODV envelope and their proposed orientation (Slack and Arif, 2007). Republished with permission.

2 Materials and Methods

2.1 Viruses and cells

Spodoptera frugiperda IPLB-Sf21-AE clonal isolate 9 (Sf9) insect cells were cultured in suspension at 27ºC in TC100 medium (Sigma Aldridge) supplemented with 10% fetal bovine serum (Invitrogen). The AcMNPV bacmid bMON14272 (Invitrogen Life Technologies) was derived from the AcMNPV strain E2 and maintained in DH10B cells as described previously (Luckow et al., 1993)

2.2 Bioinformatic analysis

Ac146 was analyzed using BLAST searches to identify potential transcriptional regulatory sequences of both *ac146* and the overlapping gene *ie1*. This information was used to design *ac146* deletions and primers for transcriptional analysis. Comparisons with baculovirus proteins and all publicly available protein and nucleic acid sequences were performed and analyzed using ClustalW to identify functional domains of *ac146*.

2.3 Transcriptional analysis

Transcriptional analysis was performed by isolating total RNA from AcMNPV (E2 strain)-infected *Spodoptera frugiperda* (Sf9) cells. Cells were infected at a multiplicity of infection (MOI – ratio of virus to cells) 5 and were collected at time points 0, 3, 6, 9, 12, 18, 24, and 48 hours post infection (hpi). At each time point 2 x 10^6 cells were collected and centrifuged (300 × *g* for 5 min). Cells were resuspended in 350µl of RLT with ß-mercaptoethanol from Qiagen RNeasy Kit® and placed at -80°C until harvesting was

completed. Total RNA was extracted from cells using the Qiagen RNeasy Kit® and stored at -80°C. RNA was quantified by nanospectrophotometry and visualization by ethidium bromide staining.

2.4 Northern Blot Hybridization

Total RNA (6 μg) was separated by electrophoresis in a 1.25% agarose gel containing 6% formaldehyde. The gel was run at 30 volts for 17 hours and the RNA was transferred to a Zeta-probe nitrocellulose membrane using 10X SSC buffer. The membrane was then dried to prepare for hybridization.

Run off transcripts, using T7 RNA polymerase, made a ^{32}P radiolabeled hybridization probe. A 593 bp PCR product was established by pairing primer 1814 (5'-TCATAATAC GACTCACTATAGGGAGTTGCACAACACTAT-3') containing a T7 polymerase promoter (underlined sequence) at the 3' end with primer 1578 (5'-GACTACGCCAACGTCAATT TATACTGCCCTAAT-3') at the 5' end. AcMNPV-E2 DNA was used as the PCR template. The PCR product was column purified followed by RNA synthesis with T7 RNA polymerase using methods described by Sambrook and Russell (2001).

The Zeta probe nitrocellulose membrane was wetted with 0.1 M sodium phosphate pH 7.2 and pre-hybridized in 0.25 M sodium phosphate pH 7.2, 7% SDS and 100 μg/ml of yeast RNA at 65ºC. The hybridization probe had a TCA count of 3.3 x10^6 cpm and was added to 10 ml of the hybridization buffer (0.25 M sodium phosphate pH 7.2, 7% SDS and 100 μg/ml of yeast RNA) and hybridized at 70°C for 17 hours. Membranes were washed with 2x SSC and with 1% SDS at 70°C twice for 30 minutes each. The blot was visualized by exposure to Perkin Elmer Multisensitive Phosphorscreens, scanned using a Cyclone Phosphor Imager

(Perkin Elmer) and analyzed with Optiquant Acquisition and Analysis Software V5.0 (Perkin Elmer).

2.5 5'- and 3'- RACE

Rapid amplification of cDNA ends (RACE) was used to map the start and stop sites for *ac146*. RNA was harvested at 6 and 24 hpi from Sf9 cells infected with AcMNPV-E2 as stated above. The 5' – and 3' – RACE were completed following the protocol of GeneRacer Kit (Invitrogen). As specified by the Invitrogen protocol the extracted RNA was dephosphorylated, the mRNA cap structure was removed and the RNA oligo provided by the GeneRacer Kit was ligated to the decapped mRNA. A dT primer was then used to reverse transcribe the mRNA. The GeneRacer 5' primer was paired with the 5' GSP1 (gene specific primer) 1744 (5' -ACATTGTATGTCGGCGGATGTTCTA -3') and with the 5' nested GSP2 1743 (5'- ATTGTTATCGTGTTCGCCATTAGGG-3') to determine the start site for *ac146*. The stop site for *ac146* was determined by pairing the 3' GSP1 1746 (5'-CCCGACAAATT CTACGCCAAGGATT -3') and the nested GSP2 1745 (5'-TGTGCAACTGGAAACCCG CTCTTCA-3') with the GeneRacer 3' primer. The PCR products were gel purified using the Qiagen gel purification kit and cloned into Topo pCR®–Blunt Cloning Kit (Invitrogen) and sequenced with the M13 Forward primer.

2.6 Construction of AcMNPV *ac146* knockout bacmid

An AcMNPV bacmid (bMON14727) was used to generate four *ac146* KO viruses by homologous recombination in *Escherichia coli*. The regions deleted in the *ac146* ORF were replaced by a 652 bp *zeocin* drug resistance gene (p2ZOp2A) as the template that also includes an EM7 transposition site. The four deletions have a common 3' end using primer

1551 (5'TACGGCTCGCATGTATAGAAACTTGTTACTATG*AATAAA*GGATCTCTGC AGCACGTGTT 3'). Primer 1551 has a synthetic polyA signal that is used for *ac145* and *ac146* which is shown in italics. The underlined portion of the primer is the end sequence homologous to the EM7-*zeocin* resistance cassette. The 5' end of each of the 4 deletions use different primer locations to generate sequentially larger deletions of the *ac146* ORF. The bacmid with the smallest deletion of *ac146* (KO1) uses the 5' primer (5'CGGGCATGCTAG CGCACACGGACAATGGACCCGACAAGACATGATAAGATACATTGATGA 3'). This deletion leaves 500 bp of the *ie1* translational start site and deletes 170 bp of the C-terminal end of the *ac146* ORF. The second smallest *ac146* ORF deletion (KO2) was incorporated using primers 1551 and 1553 (5'CAACAAGTACACTGCGCCGTTGGGATTTGTGGTA ACAGACATGATAAGATACATTGATGA 3'). KO2 leaves 309 bp of the *ie1* translational start site and deletes a 285 bp C-terminal region of the *ac146* ORF maintaining a hydrophilic domain adjacent to the EM7-*zeocin* cassette location. KO3 uses primer 1554 (5'GGATACG AAAACAGTCGGCCGATAAACATTAATCTGAGACATGATAAGATACATTGATGA 3') in conjunction with primer 1551 to delete 410 bp of the C-terminal end of the *ac146* ORF leaving 260 bp of the *ie1* translational start site. KO4 was created by pairing primer 1551 with primer 1555 (5'CTAAAACCAACGCGGTTATCGTTTATTTATTCAAATAGACAT GATAAGATACATTGATGA 3') which removes the largest number of nucleotides of the deletion mutants, 500 bp. This final deletion leaves only 170 bp upstream of the *ie1* translational start site.

The PCR products containing the EM7-*zeocin* drug resistance cassette with AcMNPV flanking sequences were synthesized and gel purified using a Qiagen Gel Purification Kit®. Recombination between the PCR products and the AcMNPV bacmid (bMON14272) was

performed using the λ phage Red recombinase method described by Datsenko and Wanner (2000). Fifty nanograms of gel-purified PCR product and 1μg of bacmid DNA were cotransformed into *E. coli* strain BW2115/pKD46. Cells were allowed to recover in 1ml of low salt LB in the 37ºC shaker for 3 hours and then were selected on LB agar plates that contained 50 μg/ml of kanamycin and 30 μg/ml of zeocin. Colonies were selected and bacmid DNA was isolated by Maxi prep Kit (Qiagen) and transformed into DH10B cells (Sharma and Schimke, 1996) that contain a helper plasmid. Cells were allowed to recover in 1ml of low salt LB in the 37ºC shaker for 4 hours and selected on LB agar plates containing 50 μg/ml of kanamycin and 10 μg/ml of tetracycline with blue white. Recombination events were confirmed by PCR using primer pairs 1574 (5'CGCCAGCTGCAAGCC TATC 3') and 520 (5'CCGGAACGGCACTGGTCAACT 3') for confirmation of the 5' EM7-zeocin cassette and 1575 (5' AGCTGCGCGAGTCTCGCTGTC 3') and 1239 (5' CTGACCGAC GCCGACCAACAC 3') at the 3' end of the EM7-zeocin gene. Electro-competent DH10B cells were generated containing all four knockout bacmids to produce repaired viruses. The method used to generate the *ac146* KO viruses was described by Hou et al. (2002), Lin and Blissard (2002), Lung et al. (2003), and Stewart et al. (2005).

2.7 Construction of knockout, rescue and positive control bacmids containing *polyhedrin* and *gfp*

Since the AcMNPV bacmid genome is polyhedrin negative each *ac146* KO virus was repaired to introduce polyhedrin and *gfp* (green fluorescent protein) using Tn7-mediated transposition transfer vector, pFAcT-GFP. The pFAcT-GFP transfer vector contains polyhedrin and a multiple cloning site between two Tn7 transposition excision sites in the knockout bacmids. To ensure there is no positional effect of deleting *ac146,* acBac146 KO

1-4 were rescued by replacing *ac146* and flanking sequence into the AcBAC genome at the polyhedrin loci. The repaired knockouts contain *ac146* plus flanking sequence that was generated using primers 1562 (5'TTTTTCTAGAGAGCACAAAAACTGCTAATTTACTG 3') and 1611 (5' TAACTCGAGCCGAATAATAAGCAGAACTAACTG 3'), digested using restriction sites *XbaI* and *XhoI*, that were synthetically incorporated into the primers, gel-purified and ligated into the pFAcT-GFP multiple cloning site. Repairs were also generated with an in frame HA-epitope tag at both the 5' and 3' ends of the *ac146* ORF. The repair fragments were subcloned into a pBS+ cloning vector and inverse PCR was used to incorporate the nine amino acid HA-epitope tag (5'- GGCGTAGTCGGGCACGTCGTAGG GGTA-3'). The pBS+ clones containing HA tagged fragments were digested with *XbaI* and *XhoI*, gel purified and ligated into the pFAcT-GFP transfer vectors.

The *polyhedrin* and *gfp*-positive AcMNPV bacmids were generated by Tn7-mediated transposition events; electro-competent DH10B cells containing transposon helper plasmid and *ac146* KO bacmid DNA were transformed with the transfer vectors described previously. Cells were recovered at 37°C in 1 ml of low salt LB for 4 h and plated onto low salt agar medium containing 50 μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline, 30 μg/ml zeocin, 100 μg/ml X-Gal, and 40 μg/ml IPTG. Plates were incubated at 37°C for 48 h. White colonies were selected and restreaked onto fresh plates for additional screening of white colonies, selected and grown as overnight cultures for Maxi preparations (Qiagen) for further PCR confirmation and use in transfection/infection assays.

2.8 Transfection of Sf9 cells with bacmid derived viral DNA

Spodoptera frugiperda (*Sf9*) insect cells were cultured in TC 100 medium supplemented with 10% fetal bovine serum to use in transfection and infection assays. *Sf9* cells were plated at 1 million per well in 6-well plates and transfected with 1.0 µg of each Qiagen® Maxi preparation bacmid construct using lipofectin. After a 4 h incubation period the transfection medium was removed and washed with 1.5 ml of Grace's medium. Cells were overlaid with 1.5 ml of TC100 medium containing 50 µg/ml of gentamicin and incubated at 27°C. The cells were observed under bright light and fluorescence microscopy at 24, 48, 72, and 96 hours post transfection (hpt). After observation the cells were harvested with a rubber policeman, using sterile conditions, the cells and supernatant were separated by centrifugation (4ºC for 3 min at 1301 x *g*) and washed in 1X PBS buffer. The supernatant was stored at 4ºC and the cells were stored at -70ºC to use for further analysis.

2.9 Plaque assay

Sf9 cells (2.0×10^6 cells/60 mm-diameter plate) were transfected with 1.0 µg of each bacmid construct (AcBAC$^{ac146\text{KO1}}$, AcBAC$^{ac146\text{KO2}}$, AcBAC$^{ac146\text{KO3}}$, AcBAC$^{ac146\text{KO4}}$, AcBAC$^{ac146\text{KO1rep}}$, AcBAC$^{ac146\text{KO1Nrep}}$, AcBAC$^{ac146\text{KO1Crep}}$, AcBAC$^{ac146\text{KO2rep}}$, AcBAC$^{ac146\text{KO2Nrep}}$, AcBAC$^{ac146\text{KO2Crep}}$, AcBAC$^{ac146\text{KO3rep}}$, AcBAC$^{ac146\text{KO3Nrep}}$, AcBAC$^{ac146\text{KO3Crep}}$, AcBAC$^{ac146\text{KO4rep}}$, AcBAC$^{ac146\text{KO4Nrep}}$, AcBAC$^{ac146\text{KO4Crep}}$, AcBAC^{GP64} and AcBAC^{WT}). After a 2 h incubation period at 27ºC, the transfection supernatant was aspirated and 1 ml of Grace's medium was added to wash the cells. An overlay of 4 ml of TC 100 medium containing 1.5% low-melting-point agarose (Seaplaque) pre-equilibrated to

37ºC was added to each plate. The dishes were then incubated at 27ºC for 8 days. The cells were observed by light and fluorescence microscopy at 48, 72, 96 hpt and 8 dpt.

2.10 Determination of IE1 expression

IE1 expression was determined using Sf9 cells transfected with AcBAC$^{ac146\text{KO 1 to 4}}$, GP64 KO and WT viruses. The transfection assay was performed in triplicate. Protein loading was controlled for by loading 20,000 cells per lane. Transfected Sf9 cells were harvested at 18 and 48 hpt and analyzed using western blot hybridization.

2.11 BV titration using quantitative real time PCR

Quantitative PCR was used to analyze budded virus production. Sf9 cells were transfected as described above, the supernatant was centrifuged (8000 x *g* for 5 min) and 100 µl of each sample were used to titer BV production. To each sample 100 µl of lysis buffer (10mM Tris-Cl pH 8.0, 100 mM EDTA, 0.5% SDS, 20 µg/ml RNase A and 80 µg/ml Proteinase K) were added and incubated overnight at 50ºC. After O/N digestion total DNA was extracted with 300 µl of phenol-chloroform, vortexed to mix and centrifuged (20 817 x *g* for 10 min). The aqueous layer was transferred to 300 µl of chloroform, vortexed to mix and centrifuged (20817 x *g* for 10 min). Again the aqueous layer was removed and diluted 1/10.

To perform quantitative PCR 2 µl of diluted DNA was added to 2X Finnzyme SYBR Master Mix (New England Biolabs) according to the manufacturer's protocol. This method amplifies a 100-bp fragment of *ac126* (chitinase) using primers 850 (5'-TTTGGCAAGGGA ACTTTGTC-3') and 851 (5'- ACAAACCTGGCAGGAGAGAG-3'). A stock, previously

titered by $TCID_{50}$ end-point dilution and qPCR, of wild-type AcMNPV (2.5×10^8 pfu/ml) diluted 10-fold was used as a standard. The samples were analyzed in a Stratagene MX4000 qPCR cycler using the following conditions: 1 cycle of 95ºC for 15 min; 40 cycles of 95ºC for 30 s, 52ºC for 24 s, 72ºC for 30s; 1 cycle of 95ºC for 1 min; 41 cycles of 55ºC for 30s. The results were analyzed by the MX4000 software (McCarthy et al., 2008; Vanarsdall et al., 2007).

2.12 Production of viable BV determined by $TCID_{50}$

$TCID_{50}$ end-point dilution in Sf9 cells was used to determine if viable BV were produced. $TCID_{50}$ was determined in triplicate at 6, 72 and 96 hpt by infecting Sf9 cells in 96-well microtiter plates. The microtiter plates were incubated at 27ºC for 5 days and analyzed by fluorescent microscopy (Chen et al., 2007; Reed and Muench, 1937).

2.13 DNA replication using quantitative real time PCR

Quantitative real time PCR was used to assess viral DNA replication as previously described by McCarthy et al. (2008) Vanarsdall et al. (2007) with some modifications. Sf9 cells were transfected, harvested and digested as described above. To differentiate between input and replicated DNA a *Dpn*I digestion was performed. Primers 1483 (5'-CGTAGTGG TAGTAATCGCCGC-3') and 1484 (5'-AGTCGAGTCGCGTCGC TTT-3') were combined with 2X Finnzyme SYBR Master Mix (New England Biolabs) and 2 µl of DNA template for qPCR analysis in a Statagene MX4000 qPCR cycler using the conditions stated above.

2.14 Time course analysis of AC146 expression

Sf9 cells (1.0×10^6 cells/35mm-diameter well of a six-well plate) were infected with BV supernatant of previously titered stock of AcBAC$^{ac146\text{KO2Nrep}}$ MOI 5. The BV stocks were titered using end-point dilution and qPCR. Cells were harvested at 6, 12, 18 24, 36, 48, 72, and 96 hpi and stored at -80ºC until further analysis.

2.15 Purification of BV and ODV

Sf9 cells were infected with AcBAC$^{ac146\text{KO2Nrep}}$ at an MOI of 0.1 in two spinners and harvested at 5 dpi. The harvested medium was centrifuged (106 x g for 10 min), supernatant was decanted and used for BV purification and the pellet was used for ODV purification. The pelleted ODV was resuspended in 10 ml of ddH_2O and sonicated for 2 min at 30% amplification. Fifty µl of 20% SDS were added and incubated for 1 h at room temperature with gentle agitation. The solution was centrifuged (1228 x g for 15 min) to pellet polyhedra, the supernatant was discarded and the pellet was resuspended in 1 ml of ddH_2O. The polyhedra were loaded onto a 40% to 59% sucrose gradient, centrifuged (956 x g for 30 min). The polyhedra band was collected at the interface, diluted to 12 ml with ddH_2O and centrifuged (956 x g for 15 min). The pellet was resuspended in 0.4 ml of ddH_2O, boiled for 15 min and allowed to cool to room temperature. Alkaline lysis was then performed by adding 3X DAS buffer (Na_2CO_3, NaCl, EDTA), incubated at 37ºC for 10 min and 1/10 v of 1 M Tris-Cl, pH 7.9, was added. The ODV were then loaded on a 35% sucrose cushion and centrifuged at 27 600 x g for 60 min in a Beckman SW 41 rotor. The pellet was resuspended in 0.2 ml of PBS with 1X protease inhibitor.

The supernatant containing the BV was centrifuged (8000 x *g* for 10 min) to remove debris, decanted and centrifuged (100 000 x *g* for 1 h at 4ºC). The pellet was resuspended in 0.2 ml of PBS and 1X protease inhibitor buffer, loaded onto a 25 to 65% sucrose gradient and centrifuged at 100 000 x g for 1 h at RT in a Beckman SW40 rotor. The BV band was collected, diluted with TE, pH 8.0 and centrifuged at 100 000 x *g* for 1 h at RT. The BV pellet was resuspended in 0.2 ml of PBS with 1X protease inhibitor.

2.16 Envelope and nucleocapsid fractionation of BV

In a 250 µl reaction, 250 µg of purified BV were incubated in 1.0% NP-40, 10 mM Tris-Cl, pH 8.5 with gentle agitation for 1 h at room temperature. The solution was loaded onto 4 ml of 30% (w/vol) glycerol/10mM Tris (pH 8.5) cushion and centrifuged 150 000 x *g* in a Beckman SW 60 rotor for 1 h at 4ºC. The pelleted nucleocapsids were dissolved in 10mM Tris (pH 7.4) with 1X protease inhibitor. The envelope proteins were recovered from the top layer of the cushion by trichloroacetic acid precipitation and resuspended in PBS with 1X protease inhibitor.

2.17 Cellular localization of AC146 by nuclear and cytoplasmic fractionation

Sf9 cells (1.0×10^6 cells/35mm-diamter well of a six-well plate) were infected with BV supernatant of previously titered stock of AcBAC$^{ac146\text{KO2Nrep}}$ MOI 5. The BV stocks were titered using end-point dilution and qPCR. Samples were harvested at 2, 24 and 48 hpi, cells were washed with 1×PBS, scraped with a rubber policeman and centrifuged (956 x *g* for 5 min). Pellets were resuspended in NP-40 lysis buffer (10 mM Tris [pH7.9], 10 mM NaCl, 5 mM MgCl2, 1mM DTT and 0.5% NP-40 [v/v]), mixed gently and incubated on ice for 5

minutes. The solution was pelleted by centrifugation (1000 × g for 3 min). The supernatant containing the cytoplasmic fraction was transferred to a fresh tube and the pellet containing the nuclei wes resuspended in NP-40 lysis buffer. Samples were stored at -20ºC until further analysis.

2.18 Western Blot

Protein samples from transfections, infections, nuclear and cytoplasmic fractionation, purified BV and ODV and nucleocapsid and envelope fractionated BV were washed twice with phosphate buffered saline (PBS), spun at 1301 x g for 3 minutes and resuspended in 2X sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer with protease inhibitor before being stored at -80°C. Protein samples were boiled for 10 min to denature and electrophoresed in 10% SDS-PAGE gels with a Bio-Rad Mini-Protean II apparatus and transferred to Millipore Immobilon-P membrane with a Bio-Rad liquid transfer apparatus. The blots were probed with one of the following primary antibodies (Ab): (i) mouse IE1 MAb, 1:5000; (ii) mouse HA II MAb, 1:1000; (iii) mouse OpMNPV VP39 MAb, 1:3000; (iv) mouse GP64 AcV5 MAb, 1:250. Horseradish peroxidase-conjugated rabbit anti-mouse secondary Ab, 1:10 000, was used with the enhanced chemiluminescence system (Perkin-Elmer).

3 Results

3.1 Bioinformatic analysis

An evaluation of all sequenced baculovirus genomes demonstrated that there are homologues of *ac146* in all group I and group II alphabaculoviruses and betabaculoviruses. No homologues of AC146 were found in the gamma – or delta – baculoviruses (Fig. 5). The conservation of a viral gene is usually indicative of a function that is generally required for baculovirus replication rather than being host or virus specific.

The expression and function of AcMNPV *ac146* is unknown; therefore, to identify potential transcriptional regulatory elements and to perform analysis of the predicted protein product bioinformatic analysis was necessary. Analysis of the upstream promoter regions of *ac146* did not identify any early gene motifs but did identify two potential late gene promoter motifs, ATAAG, located 342 and 399 bp upstream of the predicted AC146 translational start site. Two polyadenylation signals (AATAAA) were identified 299 and 345 bp downstream of the AC146 predicted translational stop site (Fig. 6). The promoter and transcriptional start site of the essential gene *ie1* is within the ORF of *ac146* and in addition the *ie0* splice acceptor site.

The predicted *ac146* ORF is 606 bp encoding a 201 amino acid protein that is 22.9 kDa. *In silico* domain structure predictions have not revealed any specific motifs that would suggest any particular function. To identify conserved and therefore potentially functional domains of AC146 a ClustalW alignment was performed (Fig. 7). The proteins shown include AcMNPV ORF146 and its homologues; *Orgyia pseudotsugata* MNPV ORF144, *Hyphantria cunea* NPV ORF11, *Helicoverpa armigera* NPV ORF13, *Lymantria dispar*

MNPV ORF16, *Mamestra configurata* NPV-A ORF163, *Cydia pomonella* GV ORF8, *Cryptophlebia leucotreta* GV ORF7, *Phthorimaea operculella* GV ORF7, *Xestia c-nigrum* GV ORF10, *Plutella xylostella* GV ORF11 and *Spodoptera litura* NPV ORF15. These homologues were chosen as representatives of the conservation observed in the alphabaculovirus and betabaculovirus. Six generally conserved regions are visible in the alignment analysis (regions I, II, III, IV, V and VI). An analysis of predicted motifs in AC146 indicated that there are 3 N-glycosylation sites [15 (NISF), 72 (NGTR) and 158 (NVSV)], 5 phosphorylation [52-55 (SGYE), 87- 93 (RLYDLFY), 109-112 (THAE), 120-122 (SVR) and 129-131 (STR)] and an N-myristoylation site [73-78 (GTRGAY)]. An evaluation of these predicted motifs indicates that one motif is conserved in all the AC146 homologues which is the phosphorylation site found at position 52 which is a lysine residue. The conservation of this region highlights its importance to the protein and also suggests that phosphorylation may be a critical component of its function. Another site that is conserved in all alphabaculoviruses is a potential electron acceptor site inferring enzyme activity found at position 117 which is a tryptophan residue. Region V has an amino acid conserved in all proteins but it does not map to known or identifiable functional motif.

3.2 *Ac146* Transcriptional analysis

Transcriptional mapping of *ac146* was performed using two methods: Northern blot analysis and 5' – and 3' – RACE. This analysis was performed to determine temporal expression of *ac146* during the viral life cycle and to identify which of the regulatory sequences predicted by bioinformatic analysis is utilized by *ac146*. Using a single stranded RNA probe the Northern blot hybridization detected a 1.2 kb and 4.1 kb bands that were

detected from 12 hpi to 48 hpi. Peak levels were detected at 24 hpi. This indicated that a*c146* is transcribed at late times infection (Fig. 8b). Further analysis by 3' - RACE showed that the *ac146* transcript terminated 299 bases down stream of the translational stop codon at the first polyadenylation signal (AATAAA) that was predicted. Sequencing results for the 5' RACE confirm that *ac146* transcription begins from the predicted ATAAG late gene motifs 399 nt upstream from the translational start site of *ac146* (Fig. 8a). The RACE results therefore mapped the *ac146* transcript to be 1288 nt which agrees with 1.2 kb mRNA observed in the Northern blot. As only a single termination site was observed by 3' RACE, the 4.1 kb mRNA mapped to *ac148* (*odv-e56*). *Odv-e56* is a known late gene that produces long transcripts during infection (Theilmann et al., 1996).

3.3 Construction of AcMNPV bacmids containing *ac146* gene knockouts

To analyze the function of AC146 in the viral life cycle it was necessary to construct *ac146* KO mutants using AcMNPV bMON14272 bacmid. Four *ac146* KO viruses were designed to take into consideration that the undefined promoter for essential gene *ie1* was located within the *ac146* ORF. It was important to delete as much of the *ac146* ORF as possible to ensure that *ac146* function was impaired without affecting IE1 expression. Based on the initial sequence analysis the four *ac146* KOs were designed and constructed with a common 3' terminus and variable 5'-termini. The deletions leave -452 bp, -336 bp, -206 bp and -116 bp relative to the transcriptional start site of *ie1* intact for *ac146* KOs 1 through 4, respectively (Fig. 9). As the entire *ac146* ORF is not deleted each of the *ac146* KOs could potentially express deleted peptides ranging in size from 32 to 145 amino acids. Each of the *ac146* KO viruses (KO1, KO2, KO3 and KO4) was repaired with four different constructs

which were inserted at the polyhedrin locus by Tn7 transposition. The four constructs were (i) *polyhedrin* and *GFP*; (ii) *polyhedrin*, *GFP and ac146*; (iii) *polyhedrin*, *GFP and ac146* tagged with an N-terminal HA epitope tag; (iv) *polyhedrin*, *GFP and ac146* tagged with a C-terminal HA epitope tag (Fig. 10). The bMON14272 bacmid was repaired with *polyhedrin* and *GFP* and is the wild-type AcBAC virus. Lastly, a control virus GP64KO which cannot spread from cell to cell but in which viral DNA replication is unaffected was constructed that has the viral fusion gene, *gp64*, deleted (Blissard and Rohrmann, 1991). The GP64KO was repaired with the *polyhedrin* and *gfp* genes.

3.4 Replication analysis of *ac146*KO and repair viruses by fluorescence microscopy

To determine the effect of deleting *ac146* from the AcMNPV genome on virus replication, all *ac146* KO and repair viruses as well as the wild type and GP64KO viruses were purified from *E. coli* and transfected into Sf9 cells. With the aid of *gfp* expression (driven by the OpMNPV *ie1* promoter), infected cells were visualized using fluorescence microscopy (Fig. 11-14). Transfection efficiency for *ac146* KO viruses 1 through 4 was similar as evaluated by observation of the number of Sf9 infected cells with GFP fluorescence at 24 hpt. From 48 to 96 hpt there was no increase compared to $AcBAC^{WT}$ in the number of *gfp* fluorescent Sf9 cells for each of the *ac146* KO viruses, indicating that *ac146* KO viruses were unable to produce BV and move from cell to cell. Repair of each of the *ac146*KO viruses with *ac146* or with *ac146* tagged at either the N - or C - terminus resulted in all cells being infected by 72 hpt similar to $AcBAC^{WT}$.

Plaque assays were used to confirm the results of the initial transfection assay which suggested that no budded virus was being produced by the *ac146*KO viruses. As a control

the *gp64*KO virus was used; this virus does not alter DNA replication but removes the ability for the virus to produce BV. Under these conditions all of the *ac146*KO viruses and GP64KO viruses produce an infection that is restricted to a single-cell. However, the repaired *ac146*KO viruses tagged or untagged produced wild-type like plaques (Fig. 15) shows AcBAC$^{ac146\text{KO2rep}}$ and AcBAC$^{ac146\text{KO2Nrep}}$ as examples illustrating that secondary infection was occurring in these viruses and AC146 was responsible for the observed phenotype.

Light microscopy images at 8 days-pt show the production of occlusion bodies (OBs) (Fig. 15) for all viruses that have the same appearance as AcBAC^{WT}. The *ac146*KO viruses only had OBs in the initially transfected Sf9 cells in contrast to the repair and wild-type viruses which had OBs present in almost all cells. The appearance of OBs in all transfected Sf9 cells demonstrates that viral replication is occurring and proceeds to very late gene expression.

3.5 IE0 and IE1 expression in *ac146*KO transfected Sf9 cells

As indicated above, the four *ac146*KO viruses were constructed as it was unknown what impact the deletion of the *ac146* ORF would have on the expression of IE1. To determine which *ac146*KO construct should be utilized to further analyze the role AC146 plays in the AcMNPV life cycle, the expression of IE0 AND IE1 was determined for each virus. Sf9 cells were transfected with *ac146*KOs 1 through 4 and the levels of IE0 and IE1 expression were evaluated by Western blot at 18 and 48 hpt (Fig. 16). The levels of IE1 and IE0 expression were compared to the levels observed in the control AcBAC^{gp64KO} virus. The results showed that AcBAC$^{ac146\text{KO1-4}}$ all appeared to have an impact on IE1 expression. IE0 expression also

appeared to be impacted by the *ac146KO* viruses but to a lesser degree than the impact visualized on IE1 expression. At early times (18 hpt) the greatest impact was observed for AcBAC$^{ac146\text{KO1}}$ and AcBAC$^{ac146\text{KO4}}$ while the expression levels for AcBAC$^{ac146\text{KO2}}$ and AcBAC$^{ac146\text{KO3}}$ were also impacted but to a lesser degree. At late times (48 hpt) it appeared that AcBAC$^{ac146\text{KO1}}$ had the greatest impact on IE1 expression. The effect AcBAC$^{ac146\text{KO4}}$ had on IE1 expression appeared to be sustained late times but with less drastic results than IE1 expression levels at 18 hpt. AcBAC$^{ac146\text{KO2}}$ showed minimal effects on IE1 expression late times as is the case with the effects seen on IE1 expression caused by AcBAC$^{ac146\text{KO3}}$. The levels of IE1 expressed from AcBAC$^{ac146\text{KO2}}$ appear to be most similar to WT levels in comparison to the other *ac146KO* viruses especially at late times. The levels of IE0 expression are reduced but to a lesser degree than the impact observed in IE1 expression for all viruses at both early and late times. However, all four viruses could be rescued by *ac146* when inserted in the *polyhedrin* locus which indicated that the restriction to a single cell was due to the loss of AC146 and not the observed impacts on IE1 expression.

The results of the transfection assays, plaque assays and Western blot detection of IE1 expression indicated that all four of the *ac146* deletions produced the same single cell phenotype which could be rescued by insertion of *ac146* at the polyhedrin locus. As all the constructs gave the same phenotype, it would suggest that any potential AC146 deletion products (Fig. 9) that are expressed are non functional. Therefore, for further analysis of AC146 the AcBAC$^{ac146\text{KO2}}$ construct was used as it has the same phenotype as the other knockout viruses but had the least effect on the expression of the IE0 AND IE1 complex. In addition, the comparison of the transfection and plaque assays showed that each of the repair constructs using native AC146 or HA-tagged at the N or C termini, rescued the knockout

viruses producing a wild-type phenotype. Therefore for all subsequent experiments AcBAC$^{ac146\text{KO2Nrep}}$ was used as to follow AC146 in the viral infection cycle.

3.6 Virus growth curve analysis

The transfection and plaque assay results demonstrate that the removal of *ac146* leads to a defect in BV production. To confirm this finding and further analyze the production of BV two methods were used: quantitative polymerase chain reaction (qPCR) and $TCID_{50}$ end-point dilution. Sf9 cells were transfected with AcBAC$^{ac146\text{KO2}}$, AcBAC$^{ac146\text{KO2Nrep}}$, AcBAC^{WT}, and AcBAC^{GP64KO} ($TCID_{50}$ only) and at various times post transfection the supernatant was collected and analyzed. The plaque assay results suggested no BV was being produced; however, it was possible that virus was being produced but it was non-infectious. Therefore, virus genome equivalents were measured initially by qPCR as it will determine virus levels regardless of infectivity. The qPCR titers were determined at 6, 12, 15, 18, 24, 36, 48, 72 and 96 hpt. For AcBAC$^{ac146\text{KO2}}$ only background levels (due to bacmid transfection) of BV were detected indicating no BV was produced (Fig 17a). AcBAC$^{ac146\text{KO2Nrep}}$ generated BV titers equivalent to AcBAC^{WT} which indicates that the defect in BV production was not due to a genomic effect at the site of *ac146* deletion. The $TCID_{50}$ end-point dilution assays were performed at 6 and 72 hpt to confirm the qPCR results and showed that no infectious BV was produced (Fig. 17b). BV were not detected in the AcBAC$^{ac146\text{KO2}}$ or in the control virus AcBAC^{GP64KO} transfected Sf9 cells confirming that no infectious BV production was occurring. AcBAC$^{ac146\text{KO2Nrep}}$ and AcBAC^{WT} produced equivalent $TCID_{50}$ levels indicating, when combined with the qPCR results, that both viruses produced equivalent levels of infectious and non-infectious BV.

3.7 Viral DNA replication was not affected by the deletion of *ac146*

The production of occlusion bodies in the initial transfection analysis in all *ac146*KO viruses (Fig.15) indicated that very late gene expression is occurring and therefore viral DNA replication appeared not to be affected. To further analyze DNA replication and to determine if there was any quantitative impact on the levels of replication, qPCR was used to compare the levels of viral DNA replication in AcBAC^{ac146KO2}, AcBAC^{ac146KO2Nrep}, AcBAC^{WT}, and AcBAC^{GP64KO} over a time course of 6, 12, 18, 24, 36, 48, 72 and 96 hpt. AcBAC^{GP64KO} is unable to produce BV and spread from cell to cell but viral DNA replication is known to be unaffected (Blissard and Rohrmann, 1991). The results showed that AcBAC^{ac146KO2Nrep} and AcBAC^{WT} levels of viral DNA replication were equivalent and increased consistently to 96 hpt, correlating with the spread of infection through BV infection (Fig 18). For AcBAC^{ac146KO2} and AcBAC^{GP64KO} viral DNA replication levels were similar to AcBAC^{WT} up to 36 hpt. After 36 hpt there was only a marginal increase which is consistent with viruses that do not produce BV and are unable to infect new cells. The results imply that viral DNA replication is unaffected by the deletion of *ac146*.

3.8 Temporal expression and cellular expression of AC146 during infection

The transcriptional mapping of *ac146* indicated that it is a late gene and the viral replication analysis indicated that BV production was eliminated when *ac146* was deleted. To further evaluate the function of *ac146* in the viral life cycle the temporal expression and cellular localization of AC146 were determined. AcBAC^{ac146KO2Nrep} infected Sf9 cells were

harvested at 6, 12, 18, 24, 36, 48, 72, and 96 hpi and analyzed by SDS-PAGE and Western blot (Fig. 19a). HA epitope tagged AC146 was first detected at 18 hpi and continues to increase through to 72 hpi. At 96 hpi AC146 remained detectable but the levels decline likely due to cell death and lysis. This result confirmed that AC146 is indeed a late gene and that there appeared to be an ongoing need for this viral protein throughout late times post infection when virus assembly was occurring.

To determine where AC146 is located within the infected cell during infection, Sf9 cells were infected with AcBAC$^{ac146\text{KO2Nrep}}$ and harvested at 6, 24 and 48 hpi, separated into nuclear and cytoplasmic fractions and analyzed by SDS-PAGE and Western blot. Results showed that AC146 was not present at 6 hpi but was present in both cytoplasmic and nuclear fractions at 24 and 48 hpi (Fig. 19b). At 48 hpi in the nuclear fraction two bands, 23 kDa and 34 kDa, were detected. This suggests that within the nucleus a portion of AC146 was undergoing a post translational modification that caused a significant shift in size.

3.9 Western blot hybridization of AC146 in purified BV and ODV

The western blot analysis of AC146 showed that this viral protein is expressed up to very late times post infection at increasing levels and is transported to the nucleus. Many viral proteins with this expression profile are destined to be structural or virion associated. To address the possibility that AC146 is associated with virions, BV and ODV were purified from Sf9 cells infected with AcBAC$^{ac146\text{KO2Nrep}}$ and analyzed by Western blot. Additionally, BV particles were biochemically fractionated into envelope and nucleocapsid fractions. The results showed that AC146 was associated with both BV and ODV (Fig. 20). Analysis of the BV fractions showed that AC146 clearly separated with the nucleocapsid and was not found

in the envelope. As a control for the purification and fractionation methods VP39 (nucleocapsid protein) and GP64 (BV-specific envelope protein) were used and found only in the appropriate samples indicating that the methods used were successful. Interestingly, in ODV two AC146 specific bands are observed at 23 kDa and 34 kDa with the latter being present at significantly higher levels. As shown in Fig. 21 the higher molecular weight band is the same size that is observed in the nucleus of infected cells at 48 hpi. However, in BV the 23 kDa band is present at higher levels. This indicates that the 34 kDa band is the dominant AC146 species in ODV but not in BV. The nucleocapsid fraction of purified BV had only one band located at the predicted size for AC146 (23kDa). The 34 kDa product of AC146 suggests that AC146 undergoes a post translational modification when it remains within the nucleus to be packaged into the ODV form of the virus.

Figures

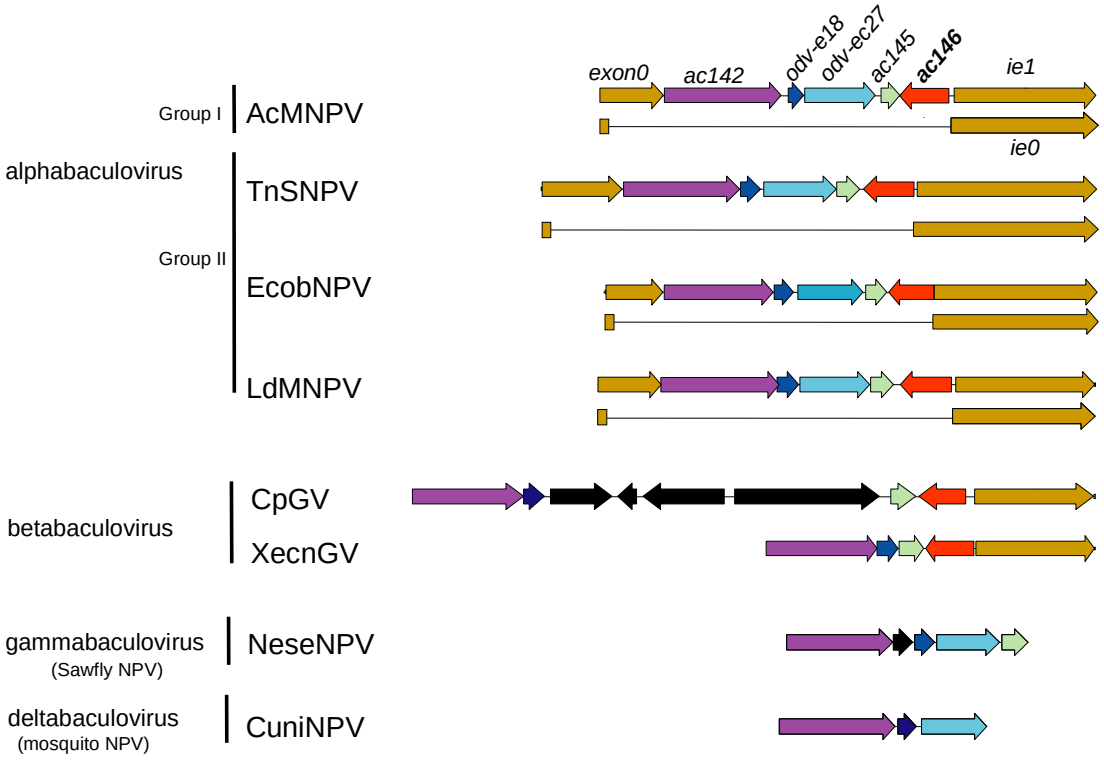

Fig. 5. Schematic representation showing the conservation of *ac146* within the baculovirus genera. The ORF is represented as a red arrow. *Ac146* is conserved in all alpha– and betabaculoviruses but no homolog has been identified in the delta – or gammabaculoviruses.

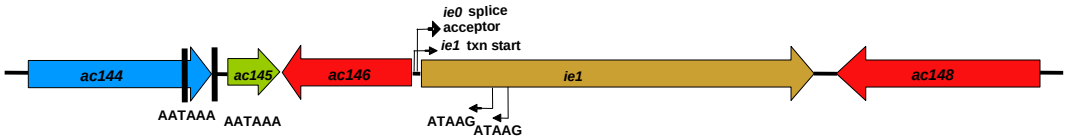

Fig. 6. Illustration showing the *ac146* loci including important motifs. The polyadenalation sites for *ac146* are shown. Also indicated are the potential late transcriptional start sites (ATAAG) for *ac146*. Important sites for the IE0 and IE1 gene complex are shown which includes the *ie1* transcriptional start site and the *ie0* splice acceptor region.

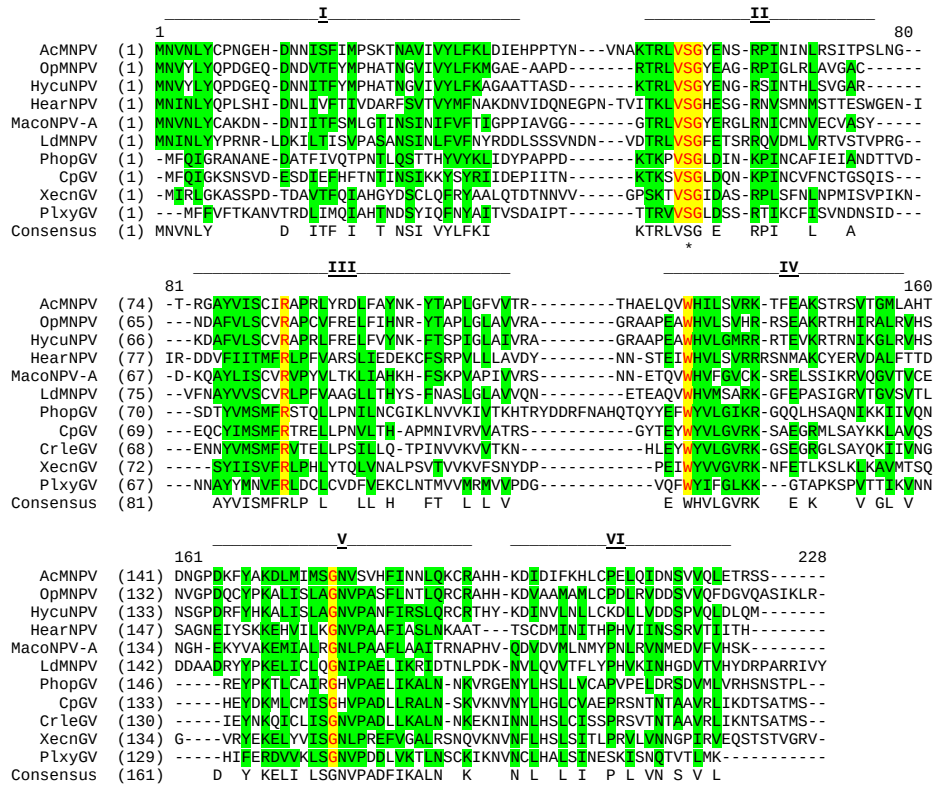

Fig. 7. Protein alignment of AC146 showing amino acid sequence conservation in the alpha- and betabaculovirus genera. The serine residue at position 52 (indicated by *) is a predicted phosphorylation site that is conserved in all alpha- and betabaculoviruses sequenced to date. The regions highlighted in yellow indicate amino acids that are identical and the areas in green represent blocks of conserved areas that have conservative changes or similar amino acids. Analyses were generated using AlignX of Vector NTI (Invitrogen, Inc) using the default similarity tables. The protein sequences used to generate the alignment profile are: NP_054177.1 for AcMNPV, NP_046300.1 for OpMNPV, YP_473199.1 for HycuNPV, NP_203570.1 for HearNPV, NP_613246.1 for MacoNPV-A, NP_047652.1 for LdMNPV, NP_663172.1 for PhopGV, NP_148792.1 for CpGV, NP_891855.1 for CrleGV, NP_059158.1 for XecnGV, and NP_068230.1 for PlxyGV.

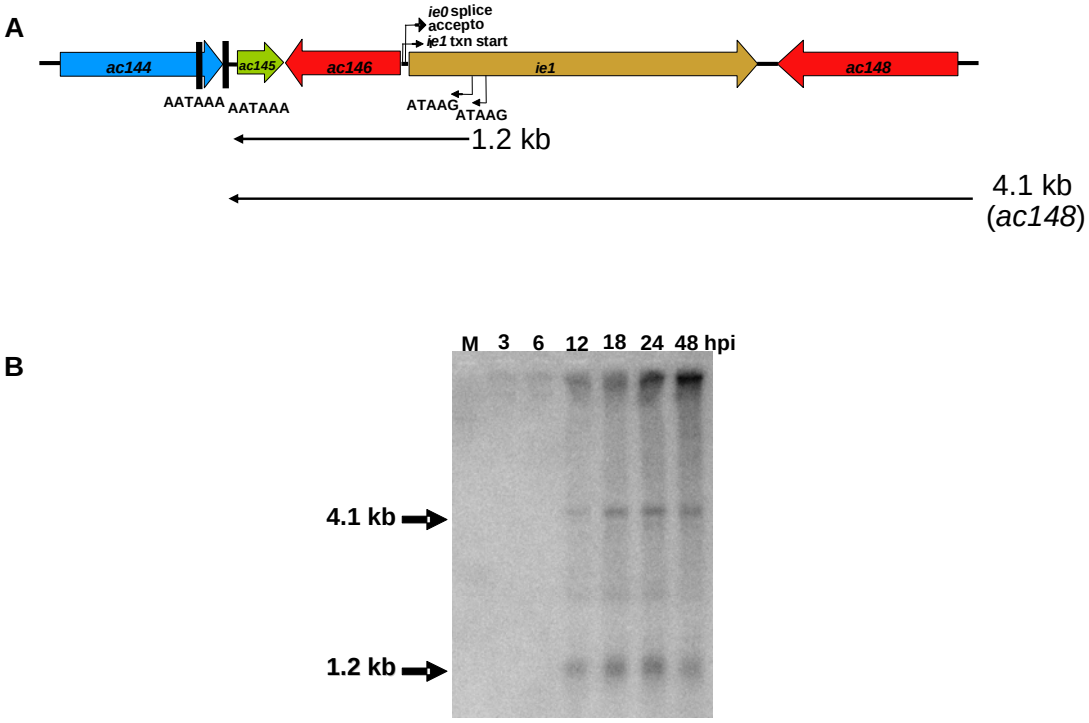

Fig. 8. Transcriptional analysis of *ac146* by Northern blot hybridization and 5'and 3' RACE. (A) Schematic representation of the sizes, positions and orientation of the *ac146* transcripts determined by Northern blot and 5' and 3' RACE analysis. (B) Northern blot analysis of *ac146* transcripts in Sf9 cells infected with AcMNPV from 3 to 48 hpi. The *ac146* specific transcript sizes are indicated on the left.

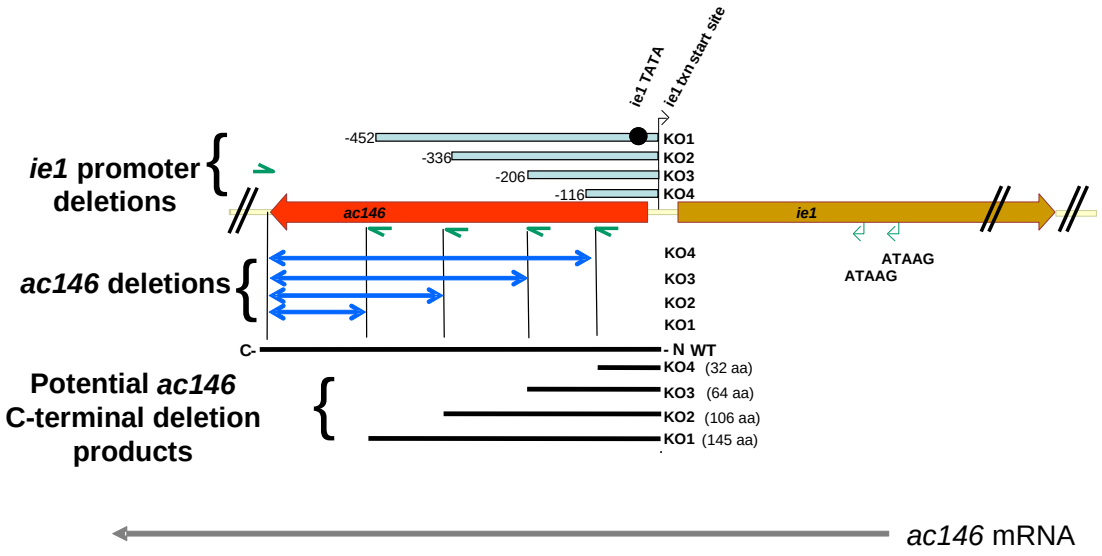

Fig. 9. A diagram illustrating the locations of the four *ac146* knockouts. Also shown is a representation of the *ie1* promoter regions (shown above ac146 ORF) remaining for each of the knockout viruses. The number of bp remaining upstream of the *ie1* transcriptional start site are shown for each virus. Also the potential *ac146* C-terminal deletion products that may be produced by each of the knockout viruses are indicated below the *ac146* deletions, these are accompanied by the number of amino acids that would remain unaffected.

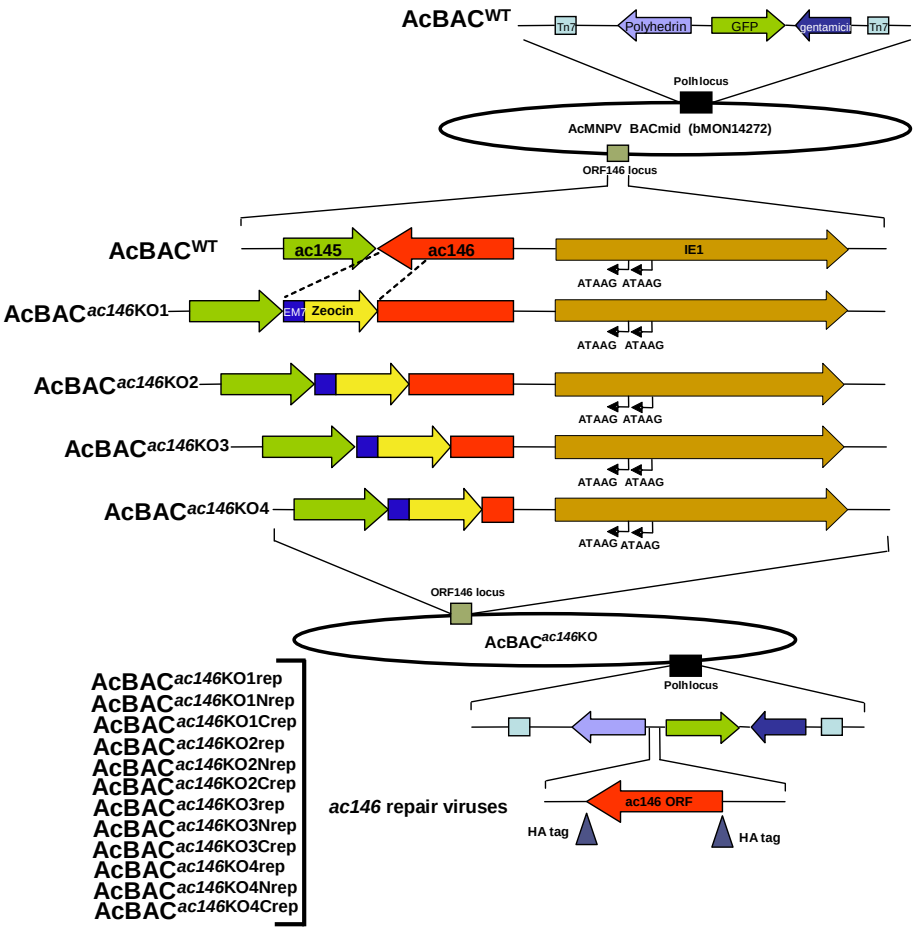

Fig. 10. Construction of the four *ac146* knockouts, twelve repair viruses and wild-type AcMNPV bacmids. The *ac146* ORF was partially deleted by replacement with an EM7-*zeocin* gene resistance cassette via homologous recombination in *E. coli*. The lower portion of the schematic illustrates the genes inserted into the polyhedrin locus to generate the *ac146* repair viruses using Tn7 mediated transposition. Each of the four ac146 KO viruses has three repair viruses: *ac146* repair virus, *ac146* N-terminal HA epitope repair virus or *ac146* C-terminal HA epitope repair virus.

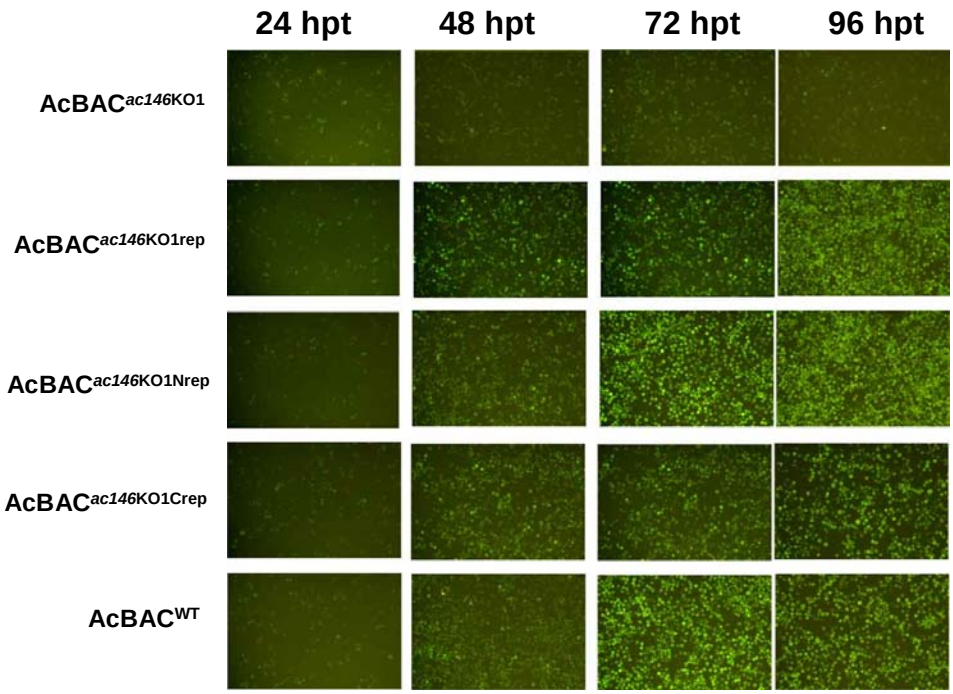

Fig. 11. Viral replication analysis of AcBAC^{ac146KO1}, AcBAC^{ac146KO1rep}, AcBAC^{ac146KO1Nrep}, AcBAC^{ac146KO1Crep} and AcBAC^{WT} viruses, with a deletion of 170 bp at the C-terminal end of the *ac146* ORF. Sf9 cells were transfected with bacmid DNA and analyzed by fluorescence microscopy at 24, 48, 72 and 96 hpt.

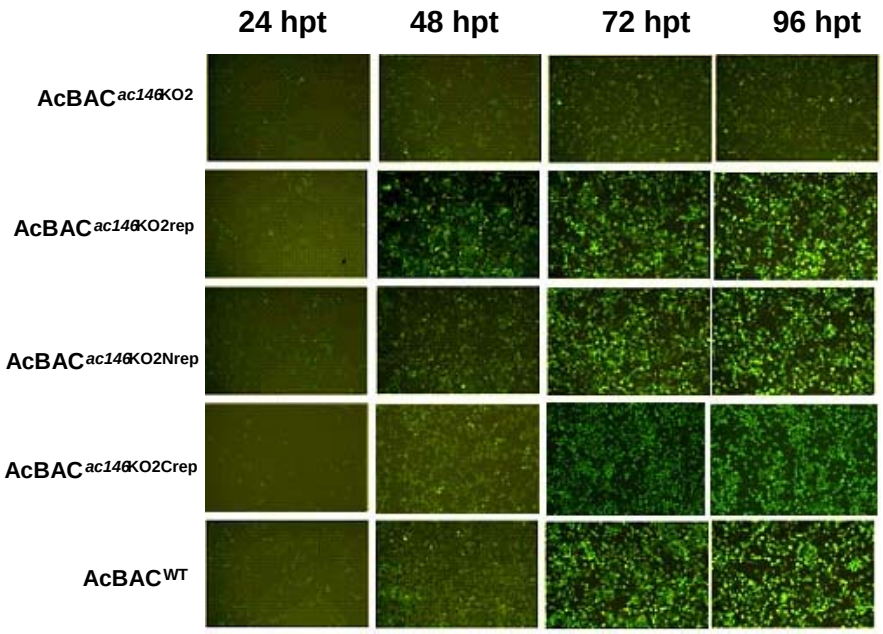

Fig. 12. Viral replication analysis of AcBAC$^{ac146\text{KO2}}$, AcBAC$^{ac146\text{KO2rep}}$, AcBAC$^{ac146\text{KO2Nrep}}$, AcBAC$^{ac146\text{KO2Crep}}$ and AcBAC$^{\text{WT}}$ viruses, with a deletion of 285 bp at the C-terminal end of the *ac146* ORF. Sf9 cells were transfected with bacmid DNA and analyzed by fluorescence microscopy at 24, 48, 72 and 96 hpt.

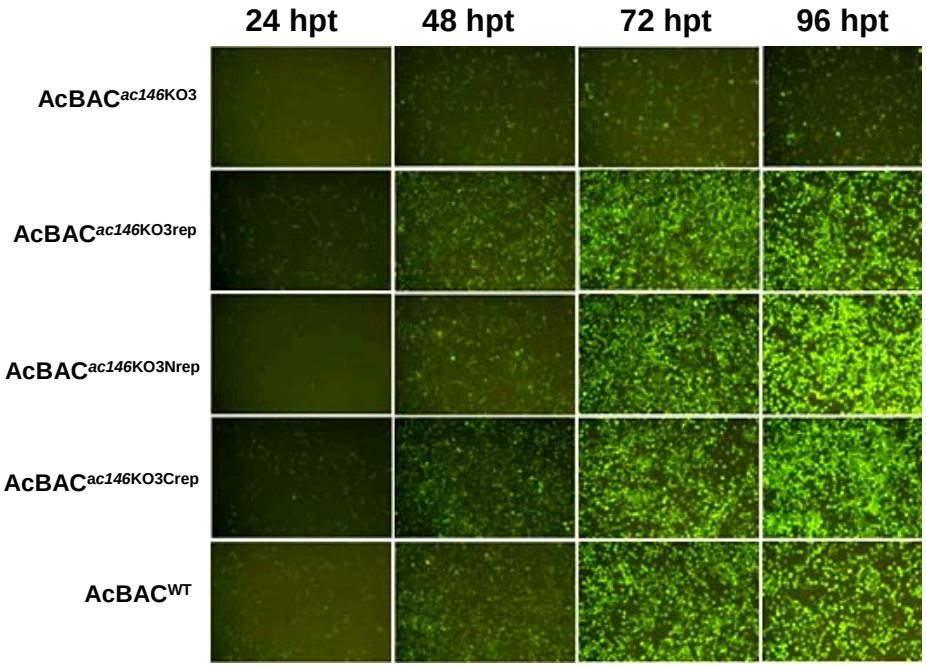

Fig. 13. Viral replication analysis of AcBAC^{ac146KO3}, AcBAC^{ac146KO3rep}, AcBAC^{ac146KO3Nrep}, AcBAC^{ac146KO3Crep} and AcBAC^{WT} viruses, with a deletion of 410bp at the C-terminal end of the *ac146* ORF. Sf9 cells were transfected with bacmid DNA and analyzed by fluorescence microscopy at 24, 48, 72 and 96 hpt.

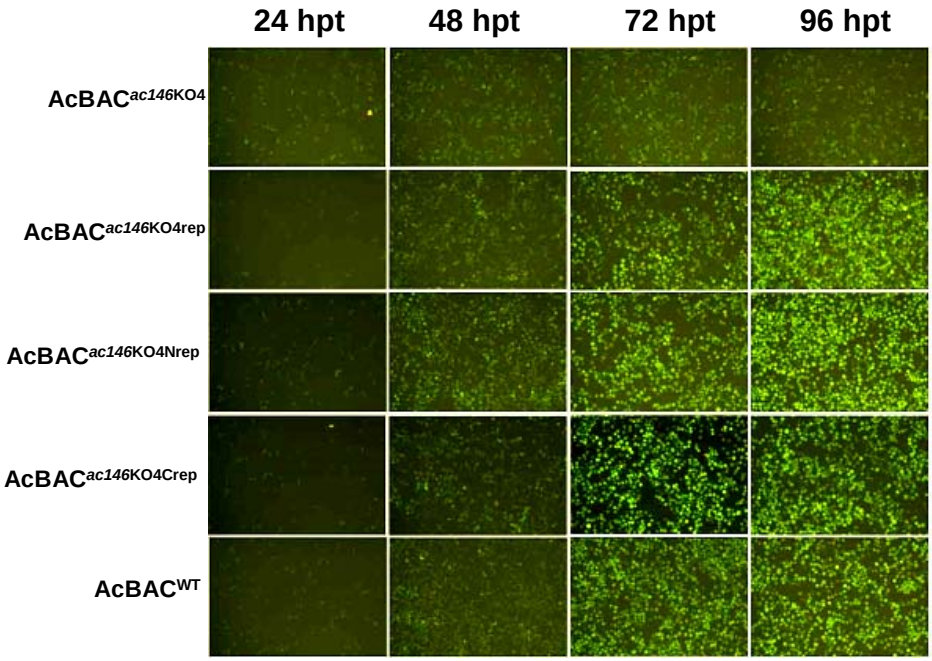

Fig. 14. Viral replication analysis of AcBAC^{ac146KO4}, AcBAC^{ac146KO4rep}, AcBAC^{ac146KO4Nrep}, AcBAC^{ac146KO4Crep} and AcBAC^{WT} viruses, with a deletion of 170 bp at the C-terminal end of the *ac146* ORF. Sf9 cells were transfected with bacmid DNA and analyzed by fluorescence microscopy at 24, 48, 72 and 96 hpt.

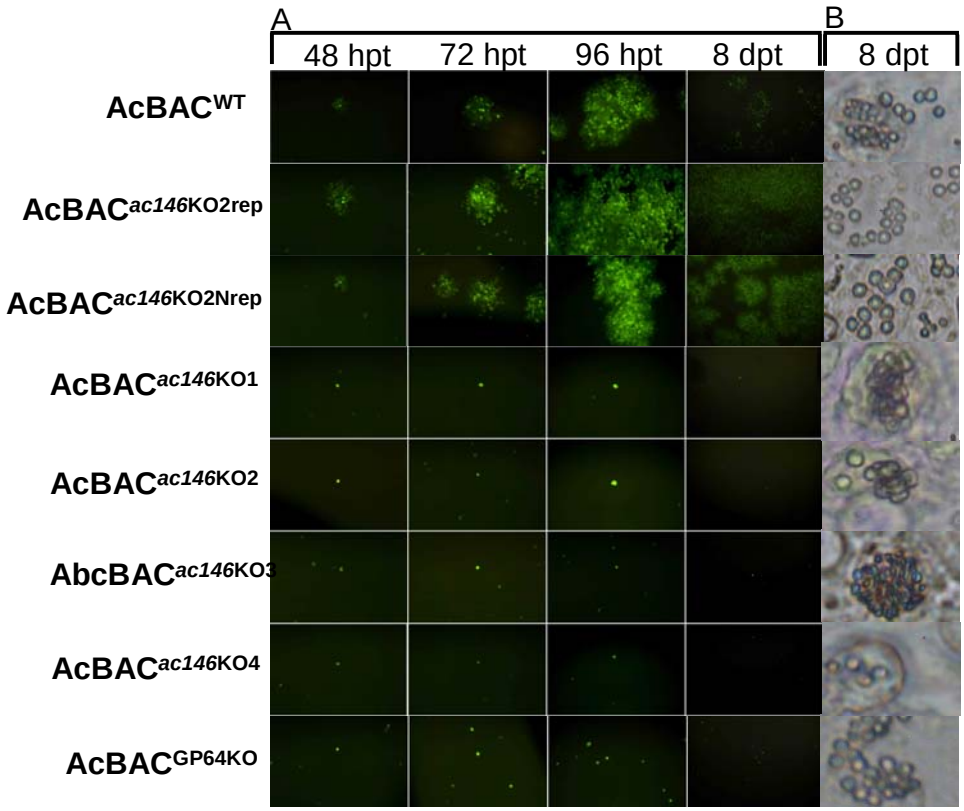

Fig. 15. Plaque assay to analyze viral replication of the *ac146* deletion viruses transfected into Sf9 cells. (A) Fluorescence microscopy at 48, 72, 96 hpt and 8 dpt showing transfected Sf9. The four ac146 knockout viruses do not produce infectious BV as is apparent by the single cell phenotype that is observed. (B) Light microscopy images of Sf9 cells transfected with *ac146* deletion mutants, AcBAC$^{ac146\text{KO2rep}}$, AcBAC$^{ac146\text{KO2Nrep}}$, and AcBAC^{WT} at 8 dpt. The occlusion bodies formed appear normal for the deletion mutants and repairs when compared to the occlusion bodies formed in AcBAC^{WT}.

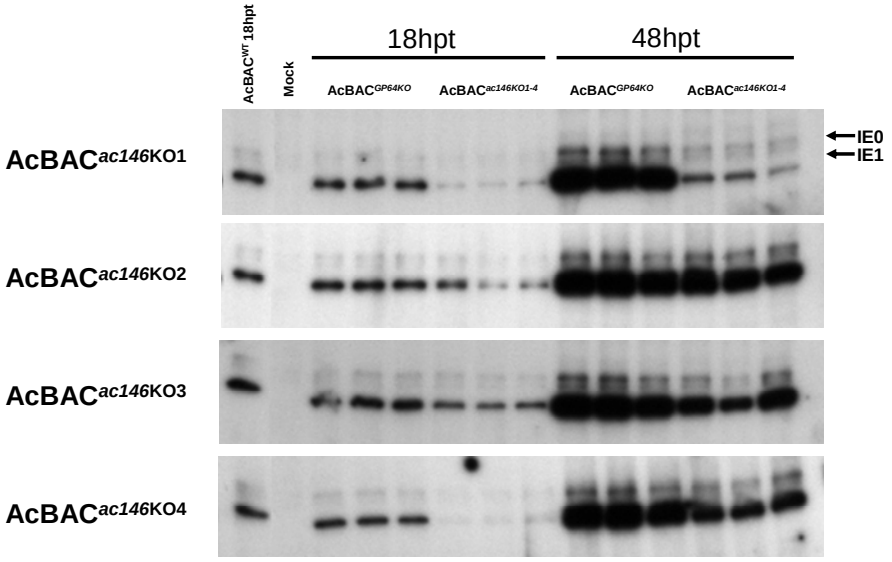

Fig. 16. Western blot analysis of IE1 and IE0 expression in AcBAC^{ac146KO1}, AcBAC^{ac146KO2}, AcBAC^{ac146KO3}, AcBAC^{ac146KO4}, AcBAC^{WT} and AcBAC^{GP64KO}. Samples were harvested at 18 and 48 hpt to analyze for the presences of IE1/IEO by western blot using anti-IE1 monoclonal antibody. IE0 AND IE1 expression for AcBAC^{GP64KO} was identical to that seen in the AcBAC^{WT} at 18 hpt. Therefore AcBAC^{GP64KO} will be used as the control for wild-type IE0 AND IE1 expression for a comparison to the AcBAC^{ac146KOs}. Expression of IE1 appears reduced in all samples at 18 hpt and 48 hpt; AcBAC^{ac146KO2} appears to show the most minimal impact in IE1 expression at late times.

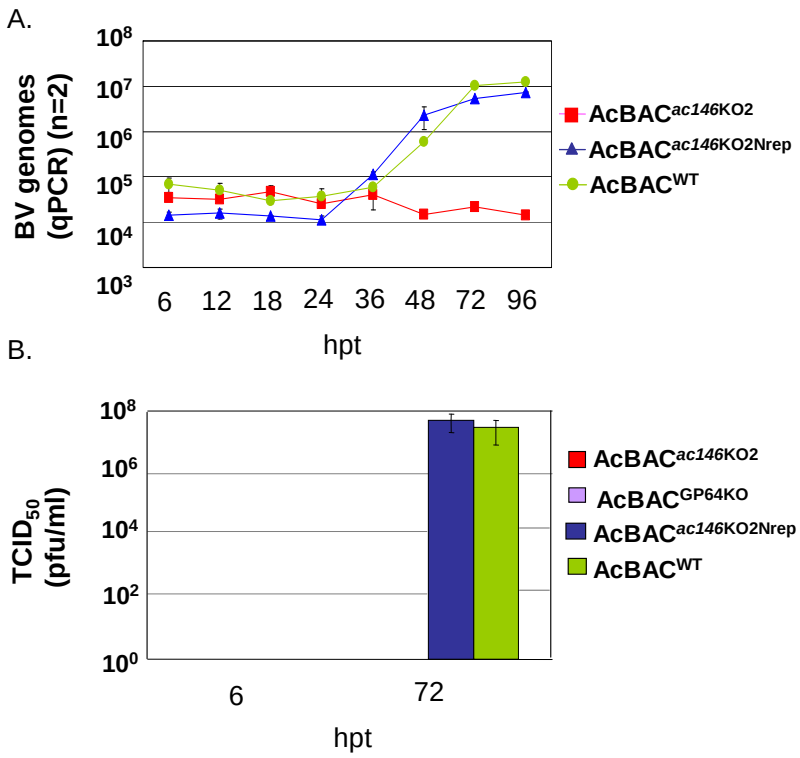

Fig. 17. Analysis of budded virus production by qPCR and $TCID_{50}$ end-point dilution. Sf9 cells were transfected with AcBAC^{ac146KO2}, AcBAC^{ac146KO2Nrep} and AcBAC^{WT} and supernatant was collected for analysis at 6, 12, 18, 24, 36, 48, 72 and 96 hpt. A) QPCR shows that *ac146* deletion mutant did not produce budded virions. B) $TCID_{50}$ end-point dilution was used to evaluate the production of infectious BV. As the graph reveals no infectious BV were produced at 72 hpt for either AcBAC^{ac146KO2} or AcBAC^{GP64KO}. The assay also indicated that the titers of infectious BV for AcBAC^{ac146KO2Nrep} and AcBAC^{WT} are similar.

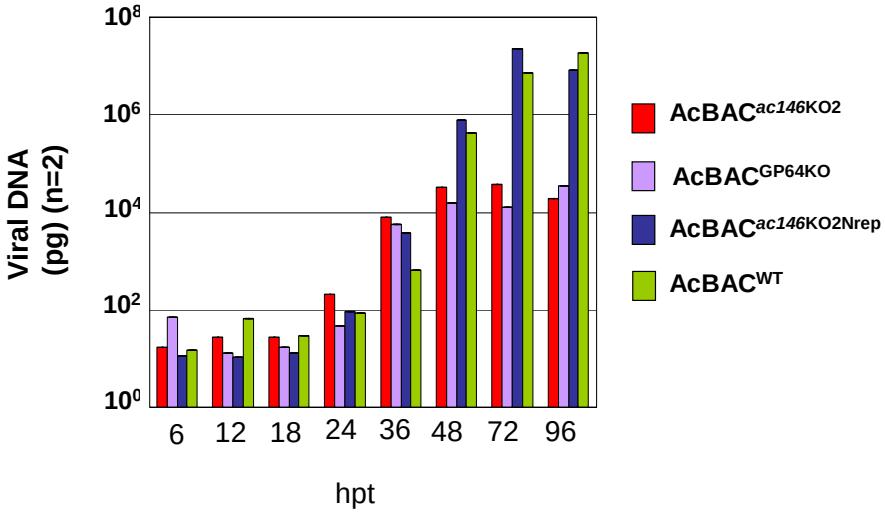

Fig. 18. Quantitative real-time PCR analysis of viral DNA replication. Sf9 cells were transfected with AcBAC^{ac146KO2}, AcBAC^{ac146KO2Nrep}, AcBAC^{GP64KO} and AcBAC^{WT} and cells were harvested over the time course indicated and analyzed. Results indicate that there was no effect on DNA replication when *ac146* was deleted compared to AcBAC^{GP64KO}. AcBAC^{GP64KO} is used as a control because it is unable to produce BV but viral DNA replication is unaffected. Error bars are too small to be seen on the scale of this graph.

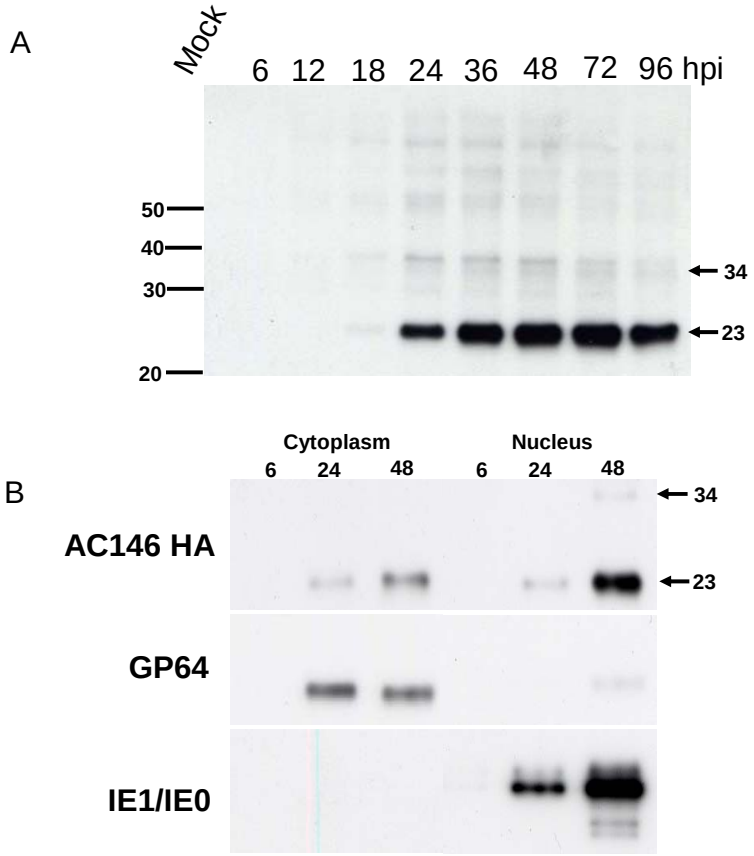

Fig. 19. Western blot analysis of the temporal expression and cellular localization of AC146. Sf9 cells were infected with AcBAC$^{ac146\text{KO2Nrep}}$ (MOI 5) and harvested at time points of 6 to 96 hpi. (A) Temporal expression of AC146 determined using anti-HA monoclonal antibody. Results indicate that AC146 has the profile of a late gene as expression of AC146 begins at 18 hpi. (B) Cellular localization of AC146 was determined by separating AcBAC$^{ac146\text{KO2Nrep}}$ infected Sf9 cells into nuclear and cytoplasmic fractions. The fractions were run on SDS-PAGE gels and probed with anti – HA, – GP64 and – IE1 monoclonal antibodies to detect HA tagged AC146, GP64 and IE0 AND IE1, respectively. The molecular weights are shown on the right in kDa.

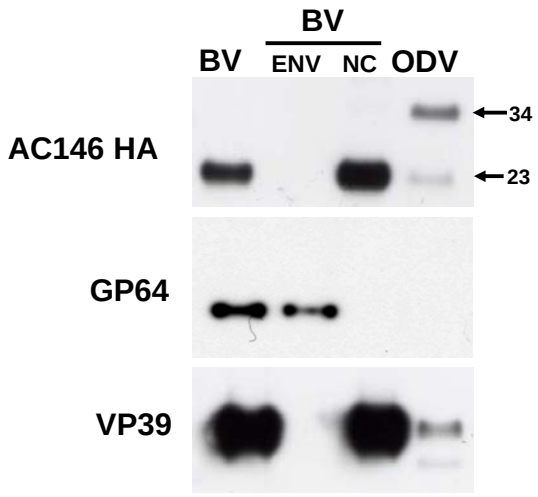

Fig. 20. Western blot analysis of AC146 in purified ODV, BV and BV envelope (ENV) and nucleocapsid (NC) fractions. AcBAC^{ac146KO2} infected (MOI 5) Sf9 cells were harvested at 96 hpi and purified by sucrose gradient into BV and ODV fractions. Subsequent fractionation of BV into ENV and NC was also performed using a sucrose gradient. AC146, GP64 and VP39 were detected using anti - HA, anti - GP64 and anti - VP39 monoclonal antibodies.

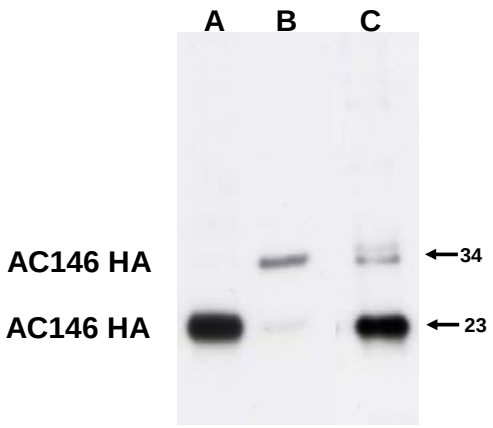

Fig. 21. A composite image illustrating the different band sizes observed for AC146 determined by Western blot analysis. Lane A, AC146 in nucleocapsid fraction of purified BV. Lane B, AC146 when found within the ODV. Lane C, AC146 located in the nuclear fraction at 48 hpi. AC146 has two products in both ODV and the nuclear fraction at late times post infection; the bands are 23 kDa and approximately 34 kDa, respectively.

4 Discussion

The conservation of AC146 extends to all sequenced baculoviruses in the alpha- and betabaculovirus genera that are known to specifically infect the order lepidoptera from the class *Insecta*. Homologs of AC146 have not been found in the genomes that have been sequenced in the delta- or gammabaculovirus which infect hymenoptera and diptera. Due to the high degree of conservation of AC146 it was hypothesized to play a key role in the baculovirus life cycle. This study confirmed this hypothesis as AC146 was shown to be an essential gene in the life cycle of AcMNPV such that the deletion of *ac146* showed no viral movement beyond initially transfected Sf9 cells.

In this study the AcMNPV gene *ac146* was extensively analyzed and transcriptional analysis of *ac146* by Northern blot analysis and 5' and 3' RACE confirmed the prediction that *ac146* is a late gene. Northern blot analysis identified two mRNAs of 1.2 and 4.1 kb homologous to the *ac146* ORF that were expressed from 12 - 48 hpi. The 1.2 kb mRNA corresponds to the size predicted by the 5' and 3' RACE. The 4.1 kb band corresponds to a transcript initiating from the transcriptional start site from the upstream gene, ODV-e56 (*ac148*) that utilizes the same transcriptional termination site as *ac146*. ODV-e56 and its homologs have been identified as late genes that are transcribed on the same strand as *ac146* (Braunagel et al., 1996a; Theilmann et al., 1996).

The role of AC146 was examined by constructing *ac146* knockout (KO) mutants using the bacmid system (Luckow et al., 1993) which also enabled the tagging of AC146 with the HA epitope. Western blot analysis revealed that it is a nucleocapsid-associated protein and therefore could be a key structural protein for baculoviruses that infect Lepidoptera.

Bioinformatic analysis of AC146 determined there were six general regions of conservation (Fig. 7) which could represent potential functional domains. The fact that these regions are highly conserved would lead to the assumption that these areas are significant for the function of AC146. The AC146 primary sequence predicts 5 phosphorylation sites, 3 N-glycosylation sites and 1 N-myristoylation site but alignment with all known AC146 homologues determined that only one phosphorylation site, ser^{56}, located in the a VSG sequence is conserved in all homologs. Phosphorylation is the most prevalent protein modification and plays an important role in cellular functions such as inducing a conformational change which in turn can control the activity of the protein (Berg et al., 2002). Two additional amino acids are conserved in all homologues which are trp^{117} and Gly^{178}. The trp^{117} is adjacent to a histidine or tyrosine and previous reports have shown that Trp-His can function as electron acceptors of enzyme active sites (Narsimha Rao et al., 1996).

Four different *ac146* knockout viruses were constructed in this study because it was unknown what impact they would have on the promoter of *ie1,*which is located within the ORF of *ac146* (Pullen and Friesen, 1995; Theilmann and Stewart, 1991). *Ie1* encodes the primary AcMNPV transregulatory factor and is essential for virus replication (Stewart et al., 2005). The transfection and plaque assay results indicate that all four *ac146* deletions display identical phenotypes such that infection is restricted to the initially transfected Sf9 cells. The four *ac146* KO mutants were constructed deleting sequentially larger portions of the undefined *ie1* promoter at -452 bp, -336 bp, -206 bp and -116 bp relative to the transcriptional start site of *ie1*. It was necessary to determine the effect of the deletions on the expression of IE1 to conclude that the single cell infection phenotype

observed was due to the deletion of *ac146* and not due to an impact on the expression of IE1. Also located in the UTR (untranslated region) of *ac146* is the splice acceptor site for *ie0* which is the only known spliced gene in the AcMNPV genome. IE0 is similar to IE1 with an addition of 54 amino acids at the N-terminal. Both proteins are essential to obtain wild type levels of viral replication (Stewart et al., 2005). Expression of IE0 AND IE1 was evaluated using Western blot analysis of Sf9 cells transfected with all four *ac146* KO viruses. The results indicate that all the *ac146* KO viruses affected the expression of IE1 and IE0. These results would confirm previous *in vitro* studies that were performed to analyze the *ie1* promoter region (Pullen and Friesen, 1995; Theilmann and Stewart, 1991). Although there appears to be an effect on IE1 expression for all *ac146* KOs the deletion that shows the least amount of effect is AcBAC$^{ac146\text{KO2}}$. It is possible for a larger deletion to have less of an impact on IE1 expression than a smaller deletion placed in the same region of the ORF. This phenomenon is possible as there are potential sites for transcription factors that can down regulate the transcription of a gene. An example of down regulation by a transcription factor can be seen in a study by Kovacs et al. (1991); this study showed that *ie1* can down regulate the transcription of *ie0*. At 48 hpt it appears that IE1 expression is recovered to near wild-type levels for AcBAC$^{ac146\text{KO2}}$. Therefore, further analysis of AC146 was performed by using AcBAC$^{ac146\text{KO2}}$.

The series of *ac146* deletion viruses allows for a novel *in vivo* analysis of the *ie1* promoter region. Previous analysis of the AcMNPV *ie1* promoter by transient analysis using cloned chloramphenicol acetyltransferase (CAT) reporter gene constructs (Pullen and Friesen, 1995) showed that -546 to -34 upstream from the TATA element was necessary for early *ie1* transcription. Another study analyzing the OpMNPV *ie1*

promoter region was performed which indicated that sequences up to -420 relative to the *ie1* transcriptional start site were required for maximal expression of *ie1* (Theilmann and Stewart, 1991). Our *in vivo* study suggests that the *ie1* promoter is located at least -452 bp relative to the *ie1* transcriptional start site. The *ac146* deletions did not alter the *ie0* splice acceptor site although there was a reduction in the expression of IE0**.** This reduction in IE0 expression is attributed to the reduction in IE1 expression. IE1 is a known transcriptional activator that is required for maximal expression of IE0.

Another factor that needed to be taken into consideration when designing the *ac146* KO viruses was that the *ac146* promoter is located within the *ie1* ORF. The *ac146* promoter was unaffected by the deletions; therefore, *ac146* KO transcripts could have been transcribed that were subsequently translated as AC146 C-terminal deletion products potentially influencing the observed phenotype and viral life cycle. However, all the deletions gave the same phenotype indicating that if any AC146 C-terminal deleted proteins are being made they are non-functional. Future studies could include the introduction of an affinity tag at the C-terminus of the remaining *ac146* ORF which would enable monitoring of any AC146 C-terminal deletion products within the cell and viral life cycle.

All four *ac146* KO viruses showed the same single cell infection phenotype in the transfection assays and AcBAC$^{ac146\text{KO2}}$ had the least effect on IE0 and IE1 expression. Consequently, AcBAC$^{ac146\text{KO2}}$ was chosen to determine the function and location of AC146 in the AcMNPV viral life cycle. Also, since both the N-terminal and C-terminal HA-tagged repairs rescued the KO mutant to wild-type levels, the N-terminal HA-tagged repair was used as a control virus.

$TCID_{50}$ endpoint dilution and qPCR assays confirmed that the deletion of *ac146* prevents the production of BV; however, viral DNA replication was not affected. Therefore the loss of BV is not due to the lack or decrease of viral DNA levels which has been previously associated with reduced BV production (Milks et al., 2003).

Western blot analysis showed that from 18 to 96 hpi AC146 was observed in both the nucleus and the cytoplasm at the predicted size of 23kDa. In addition, a second AC146-specific band migrating at 34 kDa was also observed in the nucleus (Fig. 19B). Analysis of BV and ODV showed that AC146 is a nucleocapsid protein; however, in BV, the 23 kDa is the dominant form and ODV contain mainly the 34 kDa form. From these results it is apparent that when AC146 is not incorporated into BV it remains in the nucleus and undergoes a post translational modification which appears to ensure its packaging into ODV.

The large shift in size that is observed with AC146 is unusual and suggests a major post-translational modification. The size shift, of approximately 9-10 kDa, observed in AC146 would suggest post-translational modifications such as N-glycosylation (Dutton et al., 2008) or ubiquitination (Finley and Chau, 1991). AC146 contains specific amino acid residues required for N-glycosylation (NXS/T) (Koles et al., 2007) but these sites are not conserved in the AC146 homologues. Single or monoubiquination events have been shown to direct transport of proteins to different cellular locations and also modulate protein activity and interaction (Sloper-Mould et al., 2001). The targets for monoubiquination are lysine residues (Wu et al., 2004) and there are 11 found in AC146. Other modifications that could increase the observed size of AC146 could also include O-linked glycosylation and addition of lipid moieties. Extensive phosphorylation could

affect AC146 migration in protein gels and was shown as the only conserved motif inferring possible protein modification. This conserved site was serine phosporylation found within the $V^{51}S^{52}G^{53}$ conserved region.

AC146 was shown to be a structural component of both BV and ODV through Western blot analysis. In addition, it was shown that AC146 localized to the nucleocapsid and not the envelope fractions of BV. Thus, AC146 is now included in a group of nucleocapsid proteins that are associated with both BV and ODV within the AcMNPV genome including VP1054 (Olszewski and Miller, 1997a), AC142 (McCarthy et al., 2008), AC141 (EXON0) (Fang et al., 2007), VP39 (Thiem and Miller, 1989), VFL-1 (Yang and Miller, 1998), P78/83 (Russell et al., 1997), FP25 (Braunagel et al., 1999), BV/ODV-C42 (Braunagel et al., 2001), P24 (Wolgamot et al., 1993) and P87 (Lu and Carstens, 1992). A study by Braunagel *et al*. (2003) used a number of methods including mass spectrometry in an attempt to identify all the proteins associated with ODV. However, this study did not recognize AC146 as an ODV protein. This result is not overly surprising since other known ODV-associated proteins were also missing from this study including; PCNA, VP1054, P24, BV/ODV-E26 and 38K (Wu et al., 2008). Wu *et al*. (2008) hypothesize that this may be due to the masking of low-abundance proteins by highly expressed proteins.

It is now known that BV are not produced when AC146 is deleted from the AcMNPV genome. This study also determined that AC146 was located within ODV. The ODV of the AC146 null virus was not analyzed but it is predicted that these viruses will produce ODV that are void of nucleocapsids. Further study using electron microscopy will be needed to determine the validity of this hypothesis.

4.1 Conclusions

In conclusion, this study has shown that *ac146* is an essential late gene required for the production of BV. AC146 was also shown to be a structural component of both BV and ODV. Within BV AC146 localized to the nucleocapsid fraction, deeming AC146 to be a nucleocapsid associated protein. Additional studies evaluating the function and structure of AC146 will assist in the knowledge of nucleocapsid assembly which is a key function in the baculovirus life cycle.

This study is significant in the field of baculovirology because it identified an essential late gene of the alpha- and betabaculovirus. With the discovery that AC146 is essential for BV production comes the knowledge that AC146 is a vital gene for the virulence of the virus. This study also reveals that AC146 can not be removed to improve the efficacy of AcMNPV as a biocontrol agent. Therefore, understanding the function of AC146 is essential for dissecting the molecular pathology of AcMNPV infections.

This research was performed to complete the requirements for a Master of Science degree and as such has set a major limitation of this study, time. As there was a time constraint for completion of a Master's degree there are numerous assays that can be performed to further elucidate the role of AC146 in the viral life cycle of AcMNPV which will be discussed in the next section.

4.2 Future Studies

Many questions have risen from the study of AC146 that can now be addressed. One of the key questions is what effect does deleting AC146 have on the assembly of nucleocapsids destined for packaging within ODV and BV. Based on the fact that BV are eliminated it would be predicted that the ODV will be void of nucleocapsids. To answer this question OBs from transfected cells could be isolated and analyzed for the nucleocapsids by western blot and probing for the presence of VP39. Alternatively, transmission electron microscopy would serve to answer this question in full and also potentially identify where in the assembly pathway AC146 is functioning

Another question that has developed from our research relates to determining the nature of the 34kDa band that is observed in the nuclear fraction and ODV at late times post-infection. Performing an extensive study of post-translational modifications that potentially are occurring could reveal the source of the size shift observed for AC146 in the nucleus and ODV.

With the construction of a series of four *ac146* KO viruses located within the undefined *ie1* promoter a more thorough *in vivo* study of the *ie1* promoter could be completed. This would be significant to the field of baculovirology as *ie1* is an essential early gene required for transactivating the transcription of late and very late genes and is essential for viral DNA replication.

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