DEFINING THE ROLE OF AUTOGRAPHA CALIFORNICA MULTIPLE NUCLEOPOLYHEDROVIRUS IMMEDIATE EARLY-0 AND IMMEDIATE EARLY-1 PROTEINS IN VIRAL GENOME REPLICATION AND EARLY GENE TRANSACTIVATION

by

Nadia Sokal

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Abstract

The immediate early-0 (IE0) and IE1 proteins of the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) are key transregulators in the viral replication cycle. If both proteins are absent, the virus is inactive. Either protein can support viral replication but both are required to achieve wildtype infection. Both IE0 and IE1 are involved in viral DNA replication and transcriptional transactivation of early genes. In this study, to analyze IE0 and IE1's function, *ie0, ie0*^{MtoA} or *ie1* were placed under the control of identical promoters (*ie1* or *gp64*) to achieve comparable levels of protein expression. The *ie1* promoter produced higher levels of IE0, IE0^{MtoA} and IE1 compared to the *gp64* promoter. Time course assays of infected *Spodoptera frugiperda* 9 (*Sf*9) cells allowed examination of viral DNA replication and budded virus (BV) production. The results showed that when IE0 and IE1 protein levels were high, either IE0, IE1 or IE0 and IE1 together maintained DNA replication and BV production similar to wildtype levels. However, when IE0 and IE1 protein levels were low, only when IE0 and IE1 were present together was DNA replication and BV production similar to wildtype. These results suggest that during the virus replication cycle when cellular levels of IE0 or IE1 are low, for example at the beginning of infection, the presence of both proteins results in more efficient DNA replication. Transient transactivation studies were also performed to examine IE0 and IE1's ability to activate nineteen viral early gene promoters. At low levels of IE0 and IE1 expression, a group of viral early gene promoters were found to be differentially transactivated by IE0 alone or when both IE0 and small amounts of IE1 were together. These results suggest that during early times post-infection, when

cellular levels of viral proteins are low, IE0 in the presence of a small amount of IE1 results in rapid onset of viral DNA replication and efficient transactivation of a specific set of viral early gene promoters. In context of virus infection, rapid viral replication by IE0 and IE1and transactivation of early genes by IE0 may counter the insect's defense mechanisms like sloughing and apoptosis.

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List of Abbreviations

aa	amino acid
A	alanine
AAD	acidic activation domain
AcMNPV	*Autographa californica* multiple nucleopolyhedrovirus
AD	acidic domain
ANOVA	analysis of variance
BDI	basic domain I
BDII	basic domain II
bp	base pair
BV	budded virus
^{0}C	degrees Celsius
CAT/*cat*	chloramphenicol acetyltransferase protein/*gene*
C-terminus	carboxy-terminus
Ci	Curie
DAR	downstream activation region
DBP	DNA binding protein
DE	delayed early
DNA	deoxyribonucleic acid
dpt	days post transfection
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FBS	fetal bovine serum
Fig.	Figure
g	grams in the context of mass; gravitational force in the context of centrifugation

gen^{r}	gentamicin resistance gene
GFP/*gfp*	green fluorescent protein/*gene*
^{3}H-acetyl-CoA	tritiated acetyl coenzymeA
HA	hemagglutinin
HCl	hydrochloric acid
hpi	hours post infection
hpt	hours post transfection
hr	homologous regions
IE	immediate early
IE0/*ie0*	immediate early 0 protein/*gene*
IE0^{MtoA}/*ie0*^{MtoA}	immediate early 0 with methionine changed to alanine protein/*gene*
IE1/*ie1*	immediate early 1 protein/*gene*
IPTG	isopropyl-β-D-thiogalactopyranisode
kb	kilobase
kbp	kilobase pair
kDa	kilodalton
KO	knockout
L	late
l	litre
LB	Luria broth
LEF	late expression factor
µ	micro
m	milli in context of volume metre in context of length
M	methionine in the context of protein sequence; molar in the context of concentration
MCS	multiple cloning site

min	minute
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
n	nano
nt	nucleotides
N-terminus	amino-terminus
OB	occlusion body
ODV	occlusion derived virus
OpMNPV	*Orgyia psuedotsugata* multiple nucleopolyhedrosis virus
ORF	open reading frame
ori	origin of replication
PAGE	polyacrylamide gel electrophoresis
pBS+	pBluescribe+ plasmid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIF	*per os* infectivity factor
polh	polyhedrin gene
polyA	polyadenylation sequence
PVDF	polyvinylidene fluoride
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RNaseA	ribonuclease A
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
sec	seconds
*Sf*9	*Spodoptera frugiperda* clone 9

$TCID_{50}$	50% tissue culture infective dose
TNM-FH	*Trichoplusia ni* media – Fred Hink
Tris	Tris-hydroxymethylaminomethane
UTR	untranslated region
v	virus
V	volts
VL	very late
WASP	Wiskott-Aldrich syndrome protein
WT	wildtype
Xgal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside

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1 Introduction

Baculoviruses are not pathogenic to humans, yet have a profound impact on our lives. Baculovirus specifically target arthropods, and their effects were first observed over 5000 years ago, negatively impacting the production of silk in China (reviewed in Rohrmann, 2011). Researchers began to use baculoviruses in the 1950's to control insect populations that were harmful to agricultural crops and forests (Miller, 1997).

As basic research regarding baculoviruses and its genes progressed, it was found that baculovirus genomes could be manipulated to express foreign proteins at high levels under control of the strong polyhedrin promoter. This led to the development of baculovirus expression vector systems in the 1980s (Smith et al., 1983) for high level production of proteins for research purposes and pharmaceuticals, including vaccine development. Further, recent innovations exploiting baculoviruses involve their use for gene therapy vectors as the virus can enter mammalian cells but not cause disease (Kost and Condreay, 2002). Baculoviruses have therefore proven to be a useful tool in a variety of different applications; increased knowledge regarding these viruses will allow further innovations in health care and agriculture. Baculoviruses are also an excellent model system for studying eukaryotic DNA virus replication. Clear understanding of baculovirus replication is key to further improve baculovirus applications as well as provide fundamental knowledge in important processes in eukaryotic cell biology.

This study focuses on the type *Alphabaculovirus*, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and the roles of the two main transregulatory proteins,

IE0 and IE1 in viral DNA replication and early gene expression. IE0 and IE1 are generated from the only known baculovirus spliced gene complex that produces multiple protein products (Chisholm and Henner, 1988). Many studies have shown that the *ie0-ie1* gene complex is essential and is intricately involved in the *Alphabaculovirus* life cycle (Choi and Guarino, 1995b; Dai et al., 2004b; Huijskens et al., 2004; Nagamine et al., 2006; Okano et al., 1999; Olson et al., 2001; Olson et al., 2002; Olson et al., 2003; Pathakamuri and Theilmann, 2002; Stewart et al., 2005; Taggart and Friesen, 2009; Taggart et al., 2012).

1.1 General description of baculoviruses

Baculoviridae are diverse, varying in host range, genome size, and quantity of gene products. Baculoviruses have large, double stranded, circular DNA genomes and are only known to replicate in arthropods. Genome size ranges from 82 kbp to 179 kbp. To date, there are 31 genes, known as core genes, which have homologs in every completely sequenced genome (reviewed in Rohrmann, 2011). AcMNPV, which is the subject of this study, has a 134 kbp genome and codes for 154 methionine initiated, predicted open reading frames (ORF) of 150 nt or larger (Fig. 1) (Ayres et al., 1994). The viral genome is packaged in large rod-shaped nucleocapsids which are 200-300 nm in length and approximately 30 nm in diameter. The nucleocapsid acquires an envelope either from the host's nuclear or cellular membrane (reviewed in Rohrmann, 2011).

1.2 Baculovirus taxonomy

The current baculovirus classification system separates the family *Baculoviridae* into four genera; *Alphabaculovirus*, *Betabaculovirus*, *Deltabaculovirus* and *Gammabaculovirus* (Jehle et al., 2006). *Alphabaculovirus* infect lepidoptera, and produce single (S) or multiple (M) occlusion derived virus (ODV) as well as budded virus (BV) (Jehle et al., 2006). *Alphabaculoviruses* are further separated into 2 categories, Group I and Group II, based on phylogenetic relationships, although these designations are not recognized taxonomically (Zanotto et al., 1993). *Betabaculovirus* are lepidopteran-specific and include viruses formerly known as *granuloviruses*. *Gammabaculoviruses* include hymenoptera-specific baculoviruses and *Deltabaculoviruses* include diptera-specific baculoviruses (Jehle et al., 2006).

1.3 Alphabaculovirus life cycle

1.3.1 Primary infection and entrance into the midgut cells

Alphabaculoviruses, of which AcMNPV is a member, produce two different virion phenotypes, BV and ODV (Fig. 2). ODV can exist outside of a host as they are occluded within a stable proteinaceous occlusion body (OB). Once the OB is ingested by the host, the OB matrix composed primarily of the viral protein polyhedrin is dissolved by the alkaline environment of the midgut lumen, releasing the ODV (reviewed in Rohrmann, 2011). To access the host cells, the virion must first pass through the peritrophic membrane of the midgut. The mechanism facilitating virion passage is unclear, although a combination of proteinases and enhancins play roles in host cell

entry in some baculovirus species (Hashimoto et al., 1991; Lepore et al., 1996; Wang et al., 1994). Once through the peritrophic membrane, ODV attach to the microvilli of the columnar epithelial cells that line the midgut. A combination of mechanisms allow ODV entry, including binding of the virion to the cell, interaction with a cell receptor, interaction with envelope enzymes that facilitate entry to the midgut cell and/or membrane fusion. To date, seven viral gene products termed *per os* infectivity factors (*pif*) have been found to be essential for ODV midgut infection; *p74*, *pif1*, *pif2*, *pif3*, *pif4*, *pif5* and *pif6*. All *pif*s are core genes and all encode structural components of ODV (Fang et al., 2009; Fang et al., 2006; Faulkner et al., 1997; Kikhno et al., 2002; Li et al., 2007; Nie et al., 2012; Peng et al., 2012; Piljman et al., 2003; Sparks et al., 2011).

1.3.2 Import of nucleocapsids into the nucleus

ODV nucleocapsids are transported to the nucleus via the actin cytoskeleton. Actin polymerization drives nucleocapsid intracellular motility. The capsid protein p78/83 stimulates actin nucleation with the host Arp2/3 complex. The Arp2/3 complex regulates cytoplasmic actin assembly and nucleates branched actin filaments (Goley et al., 2006). Capsid protein p78/83 contains a Wiskott-Aldrich syndrome protein (WASP) domain. The WASP domain within p78/83 binds to actin as well as the host actin-nucleating Arp2/3 complex, allowing p78/83 to act as a nucleation-promoting factor for the Arp2/3 complex (Goley et al., 2006)

Actin-based motility occurs in two phases. During transit to the nucleus, actin-based motility is employed (Ohkawa et al., 2010). The viral nucleocapsids interact with the

cytoplasmic filaments within the nuclear pore complex, which act as a docking site when the capsid is midway through the nuclear pore complex (Au and Pante, 2012). This complex undergoes rearrangement to accommodate translocation of the capsid (Au and Pante, 2012). Once in the nucleus the viral genome is uncoated and transcription is initiated by host RNA polymerase (Huh and Weaver, 1990). For AcMNPV some of the nucleocapsids entering the cell have also been shown to move directly to the cell periphery bypassing the nucleus by actin-based motility (Ohkawa et al., 2010).

1.3.3 Gene transcription

Gene transcription occurs in an ordered cascade of immediate early (IE), delayed early (DE), late (L) and very late (VL) transcription events. In general there are five functional categories for baculovirus genes which include; RNA transcription, DNA replication, structural proteins, auxiliary proteins and proteins with unknown function to date.

Early gene products include proteins essential for viral replication as well as proteins that stimulate late gene expression. For example, viral RNA polymerase is an early gene which recognizes late viral promoters that regulate transcription of essential structural genes (Yang et al., 1991). IE and DE genes are transcribed by the host RNA polymerase II, before viral DNA replication initiates. The transcripts are 5’ capped and 3’ polyadenylated (Jun-Chuan and Weaver, 1982). Early viral gene promoters resemble host gene promoters and can consist of a TATA element 25-31 bp upstream of the transcriptional start site. The deletion or replacement of the TATA element results in a large decrease of promoter activity of IE genes, indicating that it is an important

regulatory element (Blissard and Rohrmann, 1991b; Dickson and Friesen, 1991; Theilmann and Stewart, 1991). The TATA elements direct transcription initiation at the correct start site, and are involved with the transcription initiation rate. A promoter initiator motif ATCA(G/T)T(C/T) contributes to the activity of TATA promoters but is not an essential element (Miller, 1997). There are also elements downstream of the transcriptional start site that regulate expression, and have loosely been termed downstream activation regions (DAR) (Pullen and Friesen, 1995b). The 5' untranslated regions (UTR) of immediate early genes *ie1* and *gp64* have DAR which are thought to contribute to transcription by stabilizing protein interactions within the promoter region (Miller, 1997).

Late and very late gene transcription occurs after initiation of viral DNA replication. The viral RNA polymerase transcribes late and very late genes during late stages of infection (Fuchs et al., 1983; Huh and Weaver, 1990). Late genes in AcMNPV are generally transcribed between 6 to 24 hours post infection (hpi) and very late genes are transcribed between 18 to 72 hpi (Miller, 1997). Initiation of late and very late gene transcription occurs at an essential (A/T/G)TAAG motif (Morris and Miller, 1994; Ooi et al., 1989). Very late promoters are hyperexpressed, resulting in high levels of gene products being produced. The UTR of the very late gene polyhedrin is also required for very high expression levels (Ooi et al., 1989; Rankin et al., 1988). Late expression factors (LEFs) regulate late and very late gene expression. To date, 21 genes have been identified as LEFs and are generally viral IE or DE genes that optimize viral DNA replication or are directly involved in late gene transcription (Hang et al., 1995;

Huijskens et al., 2004; Passarelli and Miller, 1993a; Passarelli and Miller, 1993b; Passarelli and Miller, 1993c; Rapp et al., 1998; Yamagishi et al., 2007).

1.3.4 Viral DNA replication, nucleocapsid egress and secondary infection

Viral DNA replication and subsequent nucleocapsid assembly occur in a virogenic stroma formed in the nucleus. Some newly formed nucleocapsids transit from the nucleus to the cytoplasm and bud through the cellular membrane producing BV. BV are required for the systemic infection of the host. In midgut cells BV travel to the basal membrane and proceed to infect tracheal cells, hemocytes, fat bodies and muscle (Fig. 2).The remaining nucleocapsids eventually form ODV within the nucleus and either single or multiple nucleocapsids are enveloped per virion and are embedded within the OB polyhedrin matrix (reviewed in Rohrmann, 2011). Permissive cells become filled with OBs which are released upon cell lysis. The viral proteins chitinase and cathepsin have been shown to aid in insect cell lysis and degrade the insect cuticle (Hawtin et al., 1997).

Upon primary infection some nucleocapsids are transported directly to the basal membrane of midgut cells bypassing the nucleus and viral replication. At the basal membrane the nucleocapsids bud through the cell membrane forming BV, allowing rapid development of secondary infection. It is thought that this mechanism counteracts the insect host's defence mechanism of sloughing midgut cells (Washburn et al., 2002).

1.4 Baculovirus DNA replication

The exact mechanism of baculovirus genome replication is unknown. Evidence suggests rolling circle replication, (Oppenheimer and Volkman, 1997) though is it thought that a more complex system occurs incorporating the theta mechanism at early times and recombination at late times (Leisy and Rohrmann, 1993). Multiple origins of replication (*ori*'s) are identified in baculoviruses including homologous regions (*hr*) that are found at multiple locations around the viral genome (Ayres et al., 1994; Pearson et al., 1992), a non-*hr ori* which resembles a classic eukaryotic *ori* (Kool et al., 1994b) and early gene promoters (Wu and Carstens, 1996). As well as these defined *ori*, it has been proposed that any unwound site of the viral genome could potentially allow the binding of the replication enzyme complex, serving as an *ori* (Okano et al., 2006). The *hr* typically contain several copies of the same element. In the AcMNPV genome there are eight *hr*'s which act as *ori*'s and also act as transcription enhancers (Mikhailov, 2003).

Replication of the baculovirus genome requires the viral proteins, DNA polymerase, helicase, late expression factor-1 (LEF1), LEF2, LEF3, LEF7, LEF11, immediate-early 1 (IE1), and IE0. Three replication factors, P35, IE2 and PE38 augment DNA replication, but are not essential. Some factors exhibit contradictory evidence for being essential or solely stimulatory (Kool et al., 1994a; Kool et al., 1994c; Lu and Miller, 1995).

1.5 The *ie0-ie1* gene complex

The focus of this thesis is on the IE0 and IE1 transregulatory proteins and their involvement in viral DNA replication and early gene transactivation. The IE0 and IE1 proteins are translated from *ie0* and *ie1* mRNA transcripts which arise from the *ie0-ie1* gene complex (Fig. 3). The *ie0-ie1* gene complex is unique within the baculovirus genome as it is the only known spliced gene that is processed into multiple protein products (Chisholm and Henner, 1988). The *ie0-ie1* gene complex is composed of *exon1* and *exon2*. The *ie1* transcript is the shorter of the two transcripts at 1.9 kb, and is composed of only *exon2* (Chisholm and Henner, 1988). The *ie0* transcript is composed of *exon2* as well as *exon1*, which is located 4.2 kb upstream of *exon* 2. The *ie0* transcript contains the complete *ie1* transcript with an additional 189 bp on its 5' end, resulting in a 2.1 kb mRNA. The *ie0* and *ie1* transcripts are under control of separate promoters.

Both *ie0* and *ie1* promoters contain the consensus immediate early gene TATA-CAGT motif, upstream as well as downstream regulatory sequences (Chisholm and Henner, 1988; Pullen and Friesen, 1995a; Pullen and Friesen, 1995b). *Ie0* and *ie1* transcripts are expressed throughout the virus life cycle but at different levels. The steady state levels of the *ie0* transcript peaks at 2-4 hpi and decreases thereafter (Chisholm and Henner, 1988; Huijskens et al., 2004). The *ie1* transcript continually increases in steady levels throughout infection (Chisholm and Henner, 1988; Huijskens et al., 2004). The *ie0* and *ie1* transcript levels reflect protein expression levels indicating that IE0 is the

dominant protein early in infection prior to replication whereas IE1 is dominant during the rest of the infection cycle.

Translation of the *ie0* transcript produces both IE0 and IE1 from the *ie1* internal start codon (Theilmann et al., 2001). It is unknown if internal translation initiation is due to a leaky scanning mechanism or by an internal ribosomal entry site (Theilmann et al., 2001). Prior to this discovery, studies examining IE0 function (Kovacs et al., 1991; Pearson and Rohrmann, 1997) were unaware that the observed trans-regulatory functions were due to the presence of IE0 as well as IE1 (Theilmann et al., 2001).

1.6 IE1 structure

The *ie1* mRNA transcript contains an ORF that encodes a 582 amino acid (aa), 66.9 kDa protein (Fig. 3) (Chisholm and Henner, 1988). The N-terminus contains a combination of three domains, a classic transcriptional acidic activation domain (AAD), an AC16 binding domain, a basic domain (BDI) followed by an acidic domain (AD), which is rich in acidic amino acids (Nie et al., 2009; Olson et al., 2003; Pathakamuri and Theilmann, 2002; Slack and Blissard, 1997). The AAD has very little sequence conservation relative to other baculovirus homologs, but is rich in acidic residues, as well as contains hydrophobic and aromatic residues which are essential for transactivation (Regier et al., 1993; reviewed in Triezenberg, 1995). The first 65 amino acids of the IE1 AAD of the *Alphabaculovirus Orgyia psuedotsugata* multiple nucleopolyhedrosis virus (OpMNPV) were found to be essential for origin-specific DNA replication (Pathakamuri and Theilmann, 2002). Recently, similar results defined the

replication domain of the AcMNPV IE1 AAD as amino acids 2 to 23 (Taggart et al., 2012). Within these 23 residues, a motif was identified that resembled a cyclin-dependent phosphorylation site (TPXR/H) and amino acid substitution in this region caused loss of DNA replication activity (Taggart et al., 2012). The IE1 AAD also contains a transactivation domain which has been shown to be separable from the essential replication domain (Pathakamuri and Theilmann, 2002). A previous study of OpMNPV IE1 showed that if the AAD was deleted, resulting in loss of transactivation it could be replaced with the archetype herpes simplex virus VP16 AAD, restoring transactivation function (Forsythe et al., 1998). The AD domain (Fig. 3) has conserved sequence and in a heterologous bacterial system it activates gene expression (Slack and Blissard, 1997). However in the absence of the AAD, in the context of IE1, no activation is observed (Slack and Blissard, 1997). BDI located between the AAD and AD inhibits the function of the AD domain (Slack and Blissard, 1997). IE0 and IE1 are both able to bind to AC16, and the AC16 binding domain is located within the acidic transcriptional activation domain (Nie et al., 2009). The relative levels of IE0 and IE1 are potentially regulated by AC16 as seen in an *ac16* knockout study (Nie et al., 2009). BDI is required for enhancer-dependent transactivation and viral DNA replication (Nagamine et al., 2005; Olson et al., 2003; Taggart and Friesen, 2009). Basic domain II (BDII) and a helix-loop-helix structure are located at the IE1 C-terminus (Okano et al., 1999; Olson et al., 2002; Olson et al., 2003; Pathakamuri and Theilmann, 2002; Rodems et al., 1997; Theilmann et al., 2001). The helix-loop-helix domain mediates oligomerization and mutation within this region results in the loss of enhancer-dependent and independent

transient transactivation (Olson et al., 2001; Rodems et al., 1997), indicating that oligomers are required for transactivation of viral early promoters. Oligomerization occurs prior to nuclear entry and the BDII domain acts as the nuclear localization signal (Kaffman and O'Shea, 1999; Olson et al., 2001; Olson et al., 2002).

1.7 IE0 structure

IE0 includes the entire IE1 protein with an additional 54 amino acids fused to the N-terminus, resulting in a 636 amino acid, 72.6 kDa protein (Chisholm and Henner, 1988)(Fig. 3). The additional amino acids are derived from 38 amino acids from *exon* 1, and 16 amino acids encoded by the *ie1* 5' UTR. No specific domains or structures are predicted from the additional amino acid sequence (Nie, 2010). As previously mentioned, translation of the *ie0* transcript results in both IE0 and IE1 being produced due to an internal translation initiation site at the *ie1* start codon (Theilmann et al., 2001).

1.8 IE0 and IE1 expression profiles

Although IE0 and IE1 are similar in structure and have similar properties, some functional differences are suggested (Huijskens et al., 2004; Lu et al., 2003; Pearson and Rohrmann, 1997; Theilmann et al., 2001). Expression profiles of both IE0 and IE1 are dissimilar. Steady state levels of IE0 peaks at 2-4 hpi, before the onset of viral DNA replication, levels decrease thereafter but remain detectable up to late times post-infection (Huijskens et al., 2004). IE1 is also detected immediately upon infection and continues to increase in steady state levels until very late times post infection (Huijskens

et al., 2004). Analysis of relative densities of IE0 and IE1 post infection show that before initiation of replication, IE0 is present at higher or equal levels compared to IE1 (Huijskens et al., 2004). IE1 becomes the dominant protein after the onset of replication and is 12-fold more abundant than IE0 by 48 hpi (Huijskens et al., 2004). This suggests IE0's role during viral infection is predominant during very early times, and IE1's role during viral infection is primarily during later times.

1.9 IE0 and IE1 function in viral replication

An AcMNPV *ie0-ie1* double-knockout virus shows that the *ie0-ie1* gene locus is essential for virus replication (Stewart et al., 2005). No viral growth and no viral DNA replication are detected in the absence of both gene products (Stewart et al., 2005). Repair of the AcMNPV double-knockout virus with either *ie0,* $ie0^{MtoA}$ or *ie1*, under control of their native *ie0* and *ie1* promoters, produces viruses expressing IE0 and IE1, IE0 or IE1 respectively. The $ie0^{MtoA}$ sequence contains a mutation changing the internal ATG methionine start codon of the *ie1* ORF to an alanine (GCG), resulting in only IE0 being translated (Stewart et al., 2005). Studies with these viruses show that either IE0 or IE1 are able to support viral replication in cell culture (Stewart et al., 2005). Note that the presence of IE0 without IE1, or vice versa, is an artificial situation that does not occur in wildtype infected cells. Although viral replication is occurring in viruses expressing IE0 or IE1, it is not equivalent to wildtype virus replication levels (Stewart et al., 2005), showing that either IE0 or IE1 can support viral replication but both are required to obtain a wildtype infection. BV levels produced by viruses expressing IE0 are significantly lower than wildtype and viruses expressing only IE1 (Stewart et al.,

2005). Viruses expressing only IE0 display delayed onset of viral DNA replication, although viral DNA replication levels surpass wildtype levels at late times (Stewart et al., 2005). The repaired *ie0-ie1* knockout viruses also shows that IE0 enhances the expression of IE1, and IE1 depresses the expression level of IE0, suggesting that IE0 and IE1 are mutually antagonistic (Huijskens et al., 2004; Stewart et al., 2005).

1.10 IE0 and IE1 are able to form dimers

IE0 and IE1 form homodimers and heterodimers (Kremer and Knebel-Morsdorf, 1998; Lu et al., 2003). The three different variations of dimers may affect activation levels of viral gene transcription and levels of viral DNA replication (Stewart et al., 2005). As previously discussed, IE0 is present in greater amounts relative to IE1 at early times post infection, leading to possibly larger amounts of the IE0-IE0 homodimer (Stewart et al., 2005). Once the levels of IE1 increase, IE1-IE0 heterodimers are predicted to be formed. At late times, when the levels of IE1 supersede the levels of IE0, the IE1-IE1 dimer may become the predominant species. It is possible that the presence of these different dimers may have an impact on processes in the virus replication cycle.

1.11 IE0 and IE1 function in early gene expression

Both IE0 and IE1 are transcriptional transactivators of early and late genes (Choi and Guarino, 1995a; Choi and Guarino, 1995b; Choi and Guarino, 1995c; Guarino and Summers, 1986a; Huijskens et al., 2004; Kovacs et al., 1991; Kremer and Knebel-Morsdorf, 1998; Passarelli and Miller, 1993c). To date, the only DNA element that IE0 and IE1 have been shown to bind to are *hr* elements (Choi and Guarino, 1995a; Olson

et al., 2003), which act as enhancers of transcription (Guarino and Summers, 1986b) as well as origins of replication (Leisy and Rohrmann, 1993; Pearson et al., 1992). IE0 or IE1 can activate genes in an enhancer-dependent and independent manner (Nissen and Friesen, 1989; Rodems and Friesen, 1993; Theilmann and Stewart, 1991). IE1 also negatively regulates transcription of immediate early genes *ie2*, *ie0* and *pe38* (Carson et al., 1991; Kovacs et al., 1991; Leisy et al., 1997; Theilmann and Stewart, 1993). Although IE0 and IE1 transactivate many early genes in transient assays, there is no evidence of a specific IE0 or IE1 responsive element within the early gene promoters. This suggests that in the absence of enhancers IE0 or IE1 may activate transcription by an indirect mechanism and not bind to the promoter directly.

Microarray analysis identified 58 genes that may be specifically regulated by IE0 and IE1 (Nie, 2010). However, it is still unknown whether IE0 and IE1 activate genes to the same level, as all previous analyses of IE0 and IE1 compare the two proteins under control of their native promoters, resulting in different expression levels. Even though data indicate that there is no specific target for IE0 and IE1, one protein may be a more efficient transactivator than the other.

1.12 IE0 and IE1 function in DNA replication

As indicated above AcMNPV DNA replication requires IE0 or IE1 and LEF-1, LEF-2, LEF-3, LEF-7, LEF-11, viral DNA polymerase and helicase and can be augmented by P35, IE2 and PE38 (Kool et al., 1994a; Kool et al., 1995; Kool et al., 1994c). When IE1 binds to *hr* elements it co-localizes in nuclear structures thought to be viral replication

factories, with LEF-3 , helicase and viral DNA binding protein (DBP) which may form a scaffold for the virogenic stroma (Kawasaki et al., 2004; Nagamine et al., 2006; Okano et al., 1999). Therefore, IE0 and IE1 may be acting as origin binding proteins allowing the replisome complex to form due to binding to *hr* elements (Blissard and Rohrmann, 1991a; Choi and Guarino, 1995a; Lu and Carstens, 1993; Mikhailov, 2003; Rodems et al., 1997).

As previously mentioned, the N-terminal AAD of both AcMNPV IE1 and OpMNPV IE1 contains a domain essential for replication (Pathakamuri and Theilmann, 2002; Taggart et al., 2012). However, DNA replication is not maintained when the OpMNPV AAD is replaced with the heterologous AcMNPV AAD, indicating that this region contributes to the specificity of the virus DNA replication complex (Pathakamuri and Theilmann, 2002). In addition, some replication domain mutants are inactive for DNA replication, but remain functional for transcriptional transactivation (Pathakamuri and Theilmann, 2002; Taggart et al., 2012). This indicates that transcriptional transactivation functions and viral DNA replication functions of IE1 are independent (Pathakamuri and Theilmann, 2002; Taggart et al., 2012).

The literature suggests that IE0 and IE1 may play different roles in viral DNA replication even though they both support replication (Stewart et al., 2005). Recombinant viruses expressing only IE0 show a delay in onset of DNA replication compared to wildtype, but viral DNA actually accumulates to higher levels at later times post-infection (Stewart et al., 2005). Whereas viruses expressing only IE1 initiate DNA replication and attain levels similar to wildtype virus (Stewart et al., 2005). These experiments express IE0

and IE1 under control of their native promoters, therefore the expression levels of each protein are dissimilar (Stewart et al., 2005). The impact observed on viral DNA replication could either be indirect due to transactivation of other viral genes or directly due to differences in the viral replisome.

A recent study showed in transient assays, $IE0^{MtoA}$ was able to support replication of a plasmid containing a viral origin of replication (Luria et al., 2012). However, no comparison was made of the ability of $IE0^{MtoA}$ to transiently replicate DNA compared to IE0 or IE1.

1.13 Summary and significance of proposed study

IE0 and IE1 are the key trans-regulatory proteins of the *Alphabaculovirus* replication cycle. IE0 and IE1 are required for all aspects of the viral life cycle (Huijskens et al., 2004; Prikhod'ko and Miller, 1999; Stewart et al., 2005). However the functional differences of these two critical viral proteins are still unknown. Their roles appear to be intricately intertwined and further research is needed to fully understand the function of the only known baculovirus spliced gene.

Determining the relative roles of IE0 and IE1 in AcMNPV DNA replication and early gene expression will provide significant insight to the biochemical basis of baculovirus virulence, eukaryotic viral gene expression and viral replication. Knowing how these proteins function will also contribute to the development of baculovirus biocontrol agents, protein expression systems and other pharmaceutical applications.

1.14 Research objectives

The goal of this project was to determine the separate functions of IE0 and IE1 in AcMNPV DNA replication and early gene expression. Based on the prior research results, it is hypothesized that;

1. AcMNPV IE0 is inefficient in its ability to support viral DNA replication compared to IE1 and
2. AcMNPV IE0 is equal to IE1 in its ability to transcriptionally transactivate viral early genes.

1.15 Experimental outline

1. Production of recombinant viruses that express only IE0 or IE1 under control of identical promoters
2. Quantitative and temporal analysis of viruses expressing only IE0 or IE1 under control of identical promoters by examination of :
    a. Protein expression
    b. Viral DNA replication
    c. Budded virus production
3. Quantitative comparison of the ability of IE0 or IE1 expressed under control of identical promoters to transactivate viral early gene promoters

2 Materials and Methods

Routine molecular biology techniques including polymerase chain reaction (PCR), restriction digestion, ligation, gel electrophoresis, Western blots, and bacterial cell preparation were performed using standard procedures (Sambrook, 2001). All PCR purification, gel extraction, plasmid and bacmid DNA preparations were performed using Qiagen or VWR kit-based systems following manufacturer's directions (see Table 1).

2.1 Cells and viruses

All cell culture experiments used *Spodoptera frugiperda* clone 9 (*Sf*9) cells maintained at 27^0C in TNM-FH media prepared from Grace's insect media (Gibco Life Technologies) supplemented with Yeastolate, Lactalbumin hydrolysate and 10% fetal bovine serum (FBS). The AcMNPV bacmid bMON14272 derived from AcMNPV stain E2 (Invitrogen Life Technologies) was used to construct all AcMNPV recombinant bacmids in *Escherichia coli (E. coli)* as described previously (Datsenko and Wanner, 2000; Luckow et al., 1993). The AcMNPV recombinant bacmids were infectious and were used to transfect *Sf*9 cells to produce recombinant viruses.

2.2 Production of AcMNPV *ac146-ie1* gene knockout bacmid (AcBac$^{ac146\text{-}ie1KO}$)

The *ie0* and *ie1* genes were removed from the wildtype bacmid by constructing an *ie1* knockout bacmid as previously described (Stewart et al., 2005). The *ie1* open reading frame (ORF) was knocked out which results in the deletion of both *ie0* and *ie1* genes, eliminating the production of both IE0 and IE1. As well as *ie0* and *ie1*, *ac146* was also deleted from the wildtype bacmid (Fig. 4). Recent investigation found that *ac146* is an

essential gene and is regulated by two late promoter motifs (ATAAG), 342 and 399 bp upstream of the *ac146* translational start site and reside within the *ie1* ORF (Fig. 4) (Dickison, 2010). Therefore, deletion of the complete *ie1* ORF also eliminates AC146 production due to the absence of the two late *ac146* promoters. Therefore the complete *ac146* gene was deleted, and subsequently reintroduced in all repaired bacmids to maintain the late promoter elements. Both the *ac146* and *ie1* ORFs were knocked out from the AcMNPV bacmid bMON14272, resulting in a bacmid that did not express *ac146*, *ie0* and *ie1*. This knockout bacmid then served as a backbone for producing recombinant bacmids expressing *ie0*, $ie0^{MtoA}$ and *ie1* under control of identical promoters (Fig. 4).

Recombination events were used to replace the *ac146-ie1* locus with a PCR amplified EM7 promoter-*zeocin* resistance cassette that encoded the gene that confers zeocin drug resistance. The EM7 promoter-*zeocin* resistance cassette also contained 5' and 3' homologous flanking regions of the *ac146-ie1* locus which allowed recombination to occur in the presence of a recombinase. The plasmid p2Zop2E (Pfeifer et al., 1997) containing the EM7 promoter-*zeocin* resistance gene was used as template and 657 bp was PCR amplified with primers 1551 and 1918 (Fig. 5). Primer 1551 incorporated the flanking homologous region of the 3' end of *ac145*, a polyA signal for *ac145*, and the homologous region 5' to the EM7 promoter-*zeocin* resistance cassette. Primer 1918 incorporated the 3' flanking region of *ie1* and a polyA signal for *odv-e56* and the 3' region homologous to the EM7 promoter-*zeocin* resistance cassette. The PCR product

that contained the *zeocin* resistance cassette and AcMNPV flanking sequences was gel purified.

Recombination between the PCR product and the bacmid genome was performed using the λ Red recombinase method (Datsenko and Wanner, 2000). Briefly, *E. coli* BW25113/pKD46 cells containing AcMNPV E2 bacmid (bMON14272) and recombinase helper plasmid (pKD46) were electroporated with 100 ng of gel purified PCR product. Transformed cells were recovered in low salt media at 37°C for 2.5 hours. The AcMNPV E2 bacmid (bMON14272) contains the gene that confers kanamycin resistance (Luckow et al., 1993) and the *ac146-ie1* knockout bacmid also contains the gene that confers zeocin resistance. Therefore, LB agar plates supplemented with kanamycin (50μg/ml) and zeocin (30 μg/ml) were used to select colonies containing the *ac146-ie1* knockout bacmid. Bacmid DNA was isolated and PCR was used to confirm the deletion of *ac146-ie1* and correct recombination at the 5' and 3' ends of the *ac146-ie1* locus (Fig. 5). Primers 1574 and 520 were used to confirm the recombination event at the 5' end of the *zeocin* resistance cassette. Primers 1919 and 1920 were used to confirm the recombination event at the 3' end of the zeocin resistance cassette. The entire *zeocin* resistance cassette was confirmed by PCR using primers 1574 and 1920. One recombinant bacmid confirmed by PCR was selected and named AcBac$^{ac146\text{-}ie1\text{KO}}$. AcBac$^{ac146\text{-}ie1\text{KO}}$ was transformed into *E. coli* DH10B cells including transposase helper plasmid (pMON7124) and the cells were made electrocompetent (Sharma and Schimke, 1996).

2.3 Construction of transfer plasmid backbone: pFAcT-GFP including *ac146* or *ac146HA*

Using the pFAcT-GFP plasmid, transfer vectors were constructed that contained AC146. One vector contained the native *ac146* ORF. The second vector contained the *ac146* ORF with additional sequence coding for a hemagglutinin (HA)-epitope tag on the 3' end of the *ac146* ORF. The second *ac146HA*-tagged vector was constructed to allow confirmation that the knockout bacmid was correctly repaired with *ac146*. This was done by using an HA antibody to detect the HA epitope in Western blot analysis of bacmid transfected cells. PCR was used to amplify ac146 using AcMNPV virus strain E2 as template and primers 1936 and 1935. Primer 1936 contained a *Pst*I restriction site and the heterologous OpMNPV *op146* late promoter. Primer 1935 contained an *Xho*I restriction site and the OpMNPV *ie2* polyA signal. Primers 1936 and 1940 were used to amplify *ac146* to include a 3' HA tag. Primer 1940 contained an *Xho*I restriction site, the OpMNPV *ie2* polyA signal and sequence coding for the HA-epitope.

The PCR products were digested using *Xho*I and *Pst*I, gel purified, ligated into pFAcT-GFP and transformed into *E. coli* DH5α cells. Successful transformants were identified by selection on LB with ampicillin (50μg/ml) and were confirmed by restriction digestion. The resulting clones were named pF and pF_{HA}. The clones were digested with *Xba*I and *Pst*I for insertion of *ie0* ,$ie0^{MtoA}$ or *ie1* under control of *ie1* or *gp64* promoters.

2.4 Construction of intermediate plasmids expressing *ie0*, *ie0*^{MtoA} or *ie1* under control of the *ie1* or *gp64* promoters

Plasmids expressing *ie0, ie0*^{MtoA} or *ie1* under control of identical promoters were constructed in two stages. First, plasmids containing either the *ie1* promoter or the *gp64* promoter were made as backbones. Subsequent insertion of *ie0, ie0*^{MtoA} or *ie1* into the backbone plasmids containing the *ie1* promoter or *gp64* promoter produced six intermediate plasmids.

The *ie1* promoter and *gp64* promoter were produced by PCR amplification using pAcIE1-DT1 (Theilmann and Stewart, 1991) and AcMNPV virus strain E2 as template respectively. Primers 1932 and 1931 were used to amplify 598 bp of the *ie1* promoter. Primer 1932 contained an *EcoR*I and *Xba*I restriction sites, whereas primer 1931 contained a *BamH*I restriction site. Primers 1933 and 1934 were used to amplify 348 bp of the *gp64* promoter. Primer 1933 contained an *EcoR*I and *Xba*I restriction sites, and primer 1934 contained a *BamH*I restriction site. The *ie1* promoter and *gp64* promoter PCR products were gel purified, *BamH*I and *EcoR*I digested, ligated into pBluescribe (pBS+) and transformed into *E. coli* DH5α cells. Successful transformants were identified by restriction digestion and positive clones were chosen and named p*ie1*p and p*gp64*p.

The vectors p*ie1*p and p*gp64*p were linearized with *BamH*I and *Pst*I and PCR amplified *ie0, ie0*^{MtoA} or *ie1* ORFs were inserted. Each ORF was amplified with a common 3' primer 1937, which contained a *Pst*I restriction site and the OpMNPV *ie2* polyA signal.

To amplify both *ie0*^{MtoA} and *ie0* the 5' primer 1939 was used which contained a *BamH*I restriction site. The *ie1* gene was amplified using the 5' primer 1938, which contained a *BamH*I restriction site. The templates used to amplify *ie0, ie0*^{MtoA} and *ie1* were previously constructed clones pAc-IE01, pAc-IE0Δ (Huijskens et al., 2004) and pAcIE1-DT1 (Theilmann and Stewart, 1991) respectively.

All PCR products were gel purified and digested with *BamH*I and *Pst*I. The digested products were ligated into p*ie1*p and p*gp64*p which generated six clones, p*ie1*p-IE0, p*ie1*p-IE0^{MtoA}, p*ie1*p-IE1, p*gp64*p-IE0, p*gp64*p-IE0^{MtoA} and p*gp64*p-IE1. A positive transformant for each clone was chosen and the target cassette was sequenced.

2.5 Construction of transfer plasmids with *ie0*, *ie0*^{MtoA} or *ie1* under control of *ie1* or *gp64* promoters and *ac146* or *ac146HA*

Transfer plasmids were constructed to contain cassettes with *ac146* or *ac146HA* and *ie0, ie0*^{MtoA} or *ie1* under control of the *ie1* promoter or *gp64* promoter. The vectors, p*ie1*p-IE0, p*ie1*p-IE0^{MtoA}, p*ie1*p-IE1, p*gp64*p-IE0, p*gp64*p-IE0^{MtoA} and p*gp64*p-IE1 were digested with *XbaI* and *PstI*, and gel purified to isolate the cassettes. Each digested cassette was individually ligated to linearized pF and pF$_{HA}$, which were previously generated from the pFAcT-GFP transfer plasmid backbone. This generated twelve transfer plasmids; pF*ie1*p-IE0, pF*ie1*p-IE0^{MtoA}, pF*ie1*p-IE1, pF$_{HA}$*ie1*p-IE0, pF$_{HA}$*ie1*p-IE0^{MtoA}, pF $_{HA}$*ie1*p-IE1, pF*gp64*p-IE0, pF*gp64*p-IE0^{MtoA}, pF*gp64*p-IE1, pF$_{HA}$*gp64*p-IE0, pF$_{HA}$*gp64*p-IE0^{MtoA}, and pF$_{HA}$*gp64p*-IE1. The "$_{HA}$" denotes the virus with AC146 tagged with the HA epitope.

2.6 Construction of repaired bacmids: Repair of AcBac$^{ac146\text{-}ie1KO}$ with transfer plasmids

The twelve transfer plasmids were used to repair AcBac$^{ac146\text{-}ie1KO}$ with cassettes containing *ac146* or *ac146HA* and *ie0*, *ie0*^{MtoA} or *ie1* under control of *ie1* or *gp64* promoters. AcBac$^{ac146\text{-}ie1KO}$ was also repaired with the unaltered pFAcT-GFP transfer vector to transpose the additional transfer vector genes (*gen*^{r}, *polh*, and *gfp*) as well as the MCS, to act as a control. Each transfer plasmid was individually transformed into electrocompetent *E. coli* DH10B cells which contained AcBac$^{ac146\text{-}ie1KO}$ and the transposase helper plasmid (pMON 7124). The transfer plasmid cassettes were inserted into the polyhedrin locus by Tn7-mediated transposition events (Luckow et al., 1993). Positive transformants indicating a successful transposition event were identified by selection on LB with kanamycin (50 μg/ml), zeocin (30 μg/ml), tetracycline (10 μg/ml), gentamicin (7 μg/ml) as well as Xgal and IPTG allowing blue and white selection. Three colonies were selected for each construct and are re-streaked onto LB supplemented with the previously mentioned selection criteria but omitting tetracycline to cure the cells of the tetracycline resistant transposase helper plasmid (pMON7124). Twelve positive clones were screened, and large-scale bacmid DNA purification was performed.

2.7 Transfection of *Sf*9 cells with repaired bacmid constructs

*Sf*9 cells were transfected with each repair bacmid, wildtype bacmid and AcBac$^{ac146\text{-}ie1KO}$. The twelve repaired bacmids and wildtype bacmid were transfected to produce

recombinant budded virus stocks, as well as to confirm IE0 and IE1 protein expression. AcBac$^{ac146\text{-}ie1\text{KO}}$ was transfected to confirm absence of production of infectious virus and absence of IE0 and IE1 protein expression. Transfection assays were performed in duplicate using *Sf*9 cells cultured in TNM-FH media. *Sf*9 cells were dispensed into 6-well plates at 1 X 10^6 cells per well. Lipofectin was used to transfect 1.0 μg of each bacmid as previously described (Campbell, 1995). The cells were overlaid with the lipofectin-bacmid mixture and incubated at 27°C for 4 hours. After incubation, the lipofectin-bacmid mixture was removed and cells were washed with 1 ml of Grace's media. Cells were overlaid with TNM-FH media and incubated at 27°C. The transfected cells were observed under bright field and fluorescence microscopy. Cells and BV supernatant were harvested at various times post transfection. Virus stocks were named v*ie1*p-IE1, v*ie1*p-IE0, v*ie1*p-IE0^{MtoA}, v*gp64*p-IE1, v*gp64*p-IE0, v*gp64*p-IE0^{MtoA}, v_{HA}*ie1*p-IE1, v_{HA}*ie1*p-IE0, v_{HA}*ie1*p-IE0^{MtoA}, v_{HA}*gp64*p-IE1, v_{HA}*gp64*p-IE0, and v_{HA}*gp64*p-IE0^{MtoA} (Fig. 6). The "$_{HA}$" denotes the virus with AC146 tagged with the HA epitope.

2.8 BV stock production and BV titration by $TCID_{50}$

*Sf*9 cells transfected with wildtype bacmid as well as repaired bacmids produced budded virus to be used in infectious time course assays. BV supernatants from bacmid transfected cells were collected at 9 dpt and were used to infect 25 x 10^6 cells at an estimated multiplicity of infection (MOI) of 0.6. After 5 days at 27°C, cells and BV supernatants were collected. Cells were collected by centrifugation at 3500 rpm for 5 minutes and BV supernatants were dispensed into 5 ml aliquots and kept at 4°C.

The BV stocks were titred for use in infectious time course assays. $TCID_{50}$ end point dilution was performed in duplicate to estimate viral titre by infecting *Sf*9 cells in 96-well microtitre plates (Lo and Chao, 2004; Reed and Muench, 1938). Each well contained 2 x 10^5 *Sf*9 cells and each row of wells was infected with a 10-fold dilution of budded virus (Reed and Muench, 1938). Infected cells were kept at 27°C for 5 days and were analyzed under fluorescence microscopy for plaque formation by presence of GFP. Viral titres were calculated using Chiptitre software (Lynn, 1992).

2.9 Time course infection assays

Time course infection assays were performed to analyze the effect of IE0 and IE1 under control of identical promoters on viral replication. Four time course infection assays were performed with slight alterations as noted. In the first pair of time course infections *Sf*9 cells (3 x 10^6 cell/50 ml tube) were infected with v*ie1*p-IE1, v*ie1*p-IE0, v*ie1*p-IE0^{MtoA}, v*gp64*p-IE1, v*gp64*p-IE0, v*gp64*p-IE0^{MtoA} and WT (wildtype) virus, each at an MOI of 5 at 27^0C. The BV supernatants used to infect the *Sf*9 cells were previously titred by $TCID_{50.}$ After 1 hour of infection, cells were washed with Grace's insect media and gently resuspended in TNM-FH media for a final concentration of 3 x 10^5 cells/ml. Infected cells were kept at 27°C with agitation and 1 ml samples were collected at 3, 6, 9, 12, 20, 24, 36 and 48 hpi. Each 1 ml sample at each time point were centrifuged at 5000 g for 5 min, BV supernatants were removed and kept at 4^0C for further use. Cell pellets were washed with 1 x phosphate buffered saline (PBS), and were split for further use; 1 x 10^5 cells for Western analysis, and 1.5 x 10^5 cells for DNA replication analysis. The remainder of the cells were kept as stock at -80°C.

In the second pair of time course infections, alterations in methods were as follows. *Sf*9 cells (3 x 10^6 cells/50 ml tube) were infected with v*ie1*p-IE1, v*ie1*p-IE0, v*ie1*p-IE0^{MtoA}, v*gp64*p-IE1, v*gp64*p-IE0, v*gp64*p-IE0^{MtoA} and WT virus each at an MOI of 5 at 27^{0}C. The BV supernatants used were previously titred by qPCR. After 1 hour of infection, cells were washed with Grace's insect media and gently resuspended in TNM-FH media. Cells were immediately dispensed into 17 microtubes at 1.5 x 10^5 cells/0.5ml for each time point. One microtube corresponding to each virus was removed at time points 3, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 24, 36, and 48 hpi. Samples were centrifuged at 5000 g for 5 min, BV supernatant was removed and kept at 4^{0}C. Cell pellets were immediately resuspended in lysis buffer (10 mM TrisCl pH 8.0, 100 mM EDTA, 0.5% SDS) and kept at -80°C for later use.

2.10 Time course of viral DNA replication

DNA replication was analyzed at all the time points for recombinant viruses with *ie0*, *ie0^{MtoA}* and *ie1* under control of identical promoters. Cells were collected, DNA extracted and absolute quantity of viral genome was determined by qPCR for all time points in all four time course assays.

Frozen cell pellets (1.5 x 10^5 cells) from the first pair of time course infection assays were resuspended with a 0.4M sodium hydroxide, 125 mM EDTA solution to dissolve occlusion bodies, and were incubated at 100°C for 10 minutes. Cells were neutralized with 0.4M HCl and 5 x 10^4 cells were removed for analysis. Cells were treated with lysis buffer (10 mM TrisCl pH 8.0, 100 mM EDTA, 0.5% SDS), incubated at 37°C with

RNaseA (20 μg/ml) for 30 min, and incubated at 55°C with proteinase K (80 μg/ml) overnight. For the second pair of infectious time course assays previously treated with lysis buffer, 1.25 x 10^5 cells were removed for DNA replication analysis. The cells were incubated at 37°C with RNaseA (20 μg/ml) for 30 min, and incubated at 55°C with proteinase K (80 μg/ml) overnight. Serial dilutions of previously quantified wildtype bacmid DNA ranging from 10^1 to 10^7 genome copies were used as template for qPCR to construct a standard curve. Duplicate dilutions were prepared and were used as template for qPCR to construct a standard curve. DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1), followed by chloroform. The aqueous phase was removed and diluted 1 in 10 prior to qPCR analysis. In a 20 μl reaction volume, 2 μl of the diluted DNA extract was used as template, with primers 850 and 851 (0.5 μM of each) and 2 x DyNAmo HS mastermix (DyNAmo HS SYBR Green qPCR kit, New England Biolabs). A previously developed qPCR thermal profile was used; 1 cycle of 95°C for 15 min; 40 cycles of 95°C for 30 sec, 52°C for 24 sec, 72°C for 30 sec; 1 cycles of 95°C for 1 min: 41 cycles of 55°C for 30 sec (McCarthy and Theilmann, 2008; Vanarsdall et al., 2007). Results were analyzed using MX4000 software (Stratagene). Technical replicates were performed for each qPCR reaction.

2.11 Time course of BV production

Budded virus production was analyzed at all the time points for recombinant viruses with *ie0*, *ie0*^{MtoA} and *ie1* under control of identical promoters. Supernatant containing BV

were collected, DNA extracted and absolute quantity of viral genome was determined by qPCR for all time points in all four time course assays.

Serial dilutions of previously titred wildtype AcMNPV budded virus were made to contain 2.5×10^1 to 2.5×10^7 AcMNPV genome copies and were used as template for qPCR to construct a standard curve. For all budded virus wildtype AcMNPV dilutions and budded virus supernatants collected over the four time course assays, 100μl of budded virus supernatant was combined with 100 μl of lysis buffer (10 mM TrisCl pH 8.0, 100 mM EDTA, 0.5% SDS), incubated at 37°C with RNaseA (20 μg/ml) for 30 min, and incubated at 55°C with proteinase K (80 μg/ml) overnight. DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) as well as chloroform and qPCR was performed as in the section "2.10 Time course of DNA replication". Results were analyzed using MX4000 software (Stratagene). Technical replicates were performed for each qPCR reaction.

2.12 Transient transactivation co-transfection assays

Four independent transient transactivation assays were performed to examine IE0 and IE1's ability to transactivate viral early gene promoters. Co-transfection of plasmids containing viral early gene promoters controlling the *cat* reporter gene (Fig. 7) and transactivator plasmids expressing IE0, IE0^{MtoA} and IE1 using the *ie1* promoter (p*ie1*p-IE0, p*ie1*p-IE0^{MtoA}, and p*ie1*p-IE1), and *gp64* promoter (p*gp64*p-IE0, p*gp64*p-IE0^{MtoA} and p*gp64*p-IE1) and subsequent CAT assays were performed as follows.

*Sf*9 cells in TNM-FH media were dispensed in six-well plates at 1 x 10^6 cells/well and were incubated at 27°C overnight. Lipofectin was used to co-transfect 0.5 µg of a viral early gene promoter-*cat* plasmid and 0.5 µg of pBS+ control plasmid, GFP transfection control plasmid or 0.5 µg of transactivator plasmid; p*ie1*p-IE0, p*ie1*p-IE0^{MtoA}, p*ie1*p-IE1, p*gp64*p-IE0, p*gp64*p-IE0^{MtoA} or p*gp64*p-IE1 (Campbell, 1995; Nie, 2010). The amount of transactivator plasmid used in the transfection was determined by titration to ensure a linear response in the CAT assay. Additional preliminary titrations were performed by co-transfection of 0.5 µg of the *cat*-reporter plasmid containing the early gene promoter for *39K* and varying amount of p*gp64*p-IE0, p*gp64*p-IE0^{MtoA} or p*gp64*p-IE1 to relate amount of transfected transactivator plasmid to rate of CAT activity. The cells were overlaid with lipofectin-plasmid mixture and were incubated at 27°C for 4 hours. After incubation, the lipofectin-plasmid mixture was removed and cells were washed with 1 ml of Grace's media. Cells were overlaid with 1.5 ml of TNM-FH media and incubated at 27°C. Transfection efficiency was monitored by fluorescence microscopy indicated by the presence of GFP and cells were harvested at 48 hpt.

The transfected cells were washed with 1X PBS, scraped from the six-well plates with rubber policemen and pelleted by centrifugation at 3000 rpm for 5 min. Cell pellets were resuspended in 100 µl of 0.25M TrisCl (pH 7.8), and lysed by three cycles of freeze-thaw (-80°C to 37°C). The lysed cells were centrifuged at 10 000 rpm for 2 min and heated at 65°C for 15 min to inactivate any cellular deacetylases. Cell lysates were stored at -80°C until used for diffusion CAT-assay with ^{3}H-acetyl-CoA.

2.13 Chloramphenicol acetyl-transferase (CAT) diffusion assay with ^{3}H-acetyl-CoA

CAT assays performed were based on a previously described method (Neumann et al., 1987). Reaction buffer containing 5mM chloramphenicol, 210mM TrisCl (pH 7.8), 125 μM acetyl coenzyme A and 0.014 μCi ^{3}H-acetyl-CoA was added to 25μl of cell lysate in a scintillation vial. The amount of cell lysate used in the reaction was titrated to determine the amount to use for a linear response in the assay. Each scintillation vial containing reaction buffer and cell lysate was overlaid with 2.5 ml of toluene-based scintillation liquid (Instafluor Plus, Perkin Elmer) and the enzymatic reaction was measured using a scintillation counter (Beckman, LS-6500). To support results, replicated randomized block analysis of variance (ANOVA) followed by a TukeyHSD post hoc test were performed as advised by an experimental design statistician (Table 3 and 4) (Loeppky, 2012). Statistical significance is defined as $p<0.05$.

2.14 Protein analysis by Western blot

Western blot analysis was performed to detect protein levels in bacmid transfections, virus infections and transient transactivation co-transfections. Cell pellets were passed through a 25 gauge syringe to shear genomic DNA and boiled to denature the protein samples. For bacmid transfection and transient transactivation samples co-transfected with *ie1* promoter driven constructs, 5×10^{4} cells were loaded onto each lane of 10% gels for sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For transient transactivation samples co-transfected with *gp64* promoter driven constructs, 1×10^{6} cells were loaded onto each lane of 10% SDS-PAGE gels. For the first pair of time

course infections and 5 x 10^4 cells were loaded and for the second pair of time course infections and 5.4 x 10^3 cells were loaded onto each lane of 10% SDS-PAGE gels. SDS-PAGE gels (Laemmli, 1970) were run using the Mini Protean II system (Bio-Rad) and protein was transferred by electroblotting at 100V overnight to PVDF membranes (Millipore). All blots were probed with mouse monoclonal anti-IE1 antibody (IE1-4B7) at 1:10000 dilution (Ross and Gaurino, 1997). A second set of bacmid transfection blots was probed with a primary mouse monoclonal anti-HA epitope antibody at 1:1000 dilution. Bound antibodies were detected using a secondary peroxidase conjugated goat anti-mouse antibody at 1:10000 dilution. Blots were exposed and visualized with Western-Lightening® Plus ECL Enhanced Chemiluminescence System (Perkin-Elmer).

3 Results

3.1 Construction of an *ie0* and *ie1* knockout bacmid

To enable the investigation of the function of IE0 and IE1, initially an *ie0-ie1* knockout virus was constructed. The *ie0-ie1* knockout virus would serve as a backbone for the construction of viruses expressing *ie0*, *ie0*^{MtoA} or *ie1* under control of the *gp64* or *ie1* promoters. In order to construct viruses that expressed only *ie0*, *ie0*^{MtoA} or *ie1* under control of identical *ie1* or *gp64* promoter, a knockout bacmid was made from the AcMNPV bMON14272 bacmid. The knockout bacmid AcBac$^{ac146\text{-}ie1KO}$ deleted *ac146* and *ie1* by replacement of *ac146-ie1* with an EM7-promoter-*zeocin* cassette by homologous recombination (Fig. 4). The knockout virus was designed to completely delete the entire *ie1* ORF, which also results in the deletion of *ie0*. However, the deletion of *ie1* also impacts the gene *ac146* which is immediately upstream of the *ie1* ORF due to the deletion of *exon2*. The promoter for *ac146* is contained within the *ie1* ORF therefore deletion of the *ie1* ORF also deletes the *ac146* promoter. To account for this overlap, the complete ORFs of both *ac146* and *ie1* were deleted (Fig. 4) and the *ac146* ORF was reinserted into all repair viruses. This was required as a previous study demonstrated that AC146 is essential for production of infectious BV(Dickison et al., 2012). To ensure the adjacent ORFs, *ac145* and *pif5,* were not affected, polyA signals were included in primers 1551 and 1918 to ensure expression of these genes.

PCR was used to confirm the recombination event to show both the correct insertion of the *zeocin* resistance cassette and the absence of *ac146-ie1* (Fig. 5). Primers 1574 and

1580 were used to confirm the absence of *ac146*. The 5' and 3' recombination events were examined using two primers pairs, 1574 with 520 and 1919 with 1920 which confirmed the junction of the *zeocin* resistance cassette with *ac145* (5' end) and *pif5* (3' end). The entire *zeocin* resistance cassette was confirmed by amplification of a 657 bp fragment using primers 1574 and 1920 (Fig 5).

3.2 Construction of repair viruses

To compare the function of IE0 and IE1, the *ie0-ie1* knockout bacmid, AcBac$^{ac146\text{-}ie1\text{KO}}$, was repaired with *ie0*, *ie0*^{MtoA} and *ie1* under control of the *ie1* promoter or *gp64* promoter (Fig. 6). The promoters were chosen because they are constitutively active in insect cells (Blissard and Rohrmann, 1991b; Chisholm and Henner, 1988; Guarino and Summers, 1988). The *ie1* promoter is native to the *ie0-ie1* gene locus, and there have been conflicting reports on whether the *ie1* promoter is autoregulated by IE1 (Kovacs et al., 1991; Nie, 2010; Theilmann and Stewart, 1991). The most recent report showed no activation by IE0 or IE1 of the *ie1* promoter (Nie, 2010), however the *gp64* promoter was also chosen to construct a second set of viruses as an alternative, in case IE1 autoregulation was observed. The *gp64* promoter has not been shown to be affected by IE0 or IE1 (Nie, 2010). The repair constructs also included *ac146*, as *ac146* was also removed from AcBac$^{ac146\text{-}ie1\text{KO}}$ as indicated above. To ensure correct reinsertion of *ac146*, two sets of repairs were constructed, one set repaired with *ac146* and a second set repaired with *ac146* including a C-terminal HA tag, (*ac146HA*). The repair constructs with *ac146HA* were able to be detected by Western blot with an anti-HA antibody to confirm AC146HA protein production. Both *ac146* and *ac146HA* were placed under

control of the OpMNPV *pif5* late promoter, and also contained the OpMNPV *ie2* polyA sequence. It has recently been confirmed that the transfer vector pFAcT-GFP including AC146HA under control of the *op146* promoter and *ie2* polyA successfully repairs AC146 knockout virus, restoring budded virus production (Dickison et al., 2012).

The repair constructs containing the *ie0*, *ie0*^{MtoA} or *ie1* ORFs and *ac146* or *ac146HA* ORFs, were inserted into the pFAcT-GFP transfer vector to facilitate repair of AcBac$^{ac146\text{-}ie1\text{KO}}$ (Dai et al., 2004a). The transfer vector pFAcT-GFP contains the *polyhedrin* (*polh*) gene, the *green fluorescence protein* (*gfp*) gene and the *gentamicin* resistance (*gen*^{r}) gene which are inserted into the bacmid AcBac$^{ac146\text{-}ie1\text{KO}}$ upon transposition (Fig. 6). AcBac$^{ac146\text{-}ie1\text{KO}}$ constructed from AcMNPV E2 bacmid strain bMON14272 is polyhedrin negative. Therefore successful transposition with the transfer vector yielded bacmid clones that were gentamicin resistant in bacteria and viruses expressed GFP and polyhedrin protein in insect cells (Dai et al., 2004a).

3.3 Fluorescence microscopy of bacmid transfected *Sf*9 cells to detect BV production

The infection generated by the constructed bacmids was initially compared by transfection and spread of virus was monitored by fluorescence microscopy. The spread of GFP signal indicated the production of infectious BV showing that IE0, IE0^{MtoA} or IE1 and AC146 or AC146HA were being expressed and could support virus replication.

AcBac$^{ac146\text{-}ie1\text{KO}}$ was examined at 24 hpt and showed single infected cells and no spread of infection was seen at 48 hpt (Fig. 8 and 9). This confirms that *ie0* or *ie1* are required

for BV production as previously shown (Stewart et al., 2005). Examination of the repaired bacmids showed spread of GFP expression indicating that all repaired bacmids produced infectious budded virus and that IE0, IE0^{MtoA} or IE1 and AC146 or AC146HA were expressed. Differences in spread of virus infection may suggest different levels of BV were produced. The viruses produced by bacmids were named to denote the genes expressed; v*ie1*p-IE0, v*ie1*p-IE0^{MtoA}, v*ie1*p-IE1, v$_{HA}$*ie1*p-IE0, v$_{HA}$*ie1*p-IE0^{MtoA}, v$_{HA}$*ie1*p-IE1, v*gp64*p-IE0, v*gp64*p-IE0^{MtoA}, v*gp64*p-IE1, v$_{HA}$*gp64*p-IE0, v$_{HA}$*gp64*p-IE0^{MtoA}, and v$_{HA}$*gp64*p-IE1. The "$_{HA}$" denotes the virus with AC146 tagged with the HA epitope.

3.4 Western blot analysis of bacmid transfected *Sf*9 cells

Observation of virus spread under fluorescence microscopy suggested that all repaired bacmids generated replicating viruses that produced BV. To analyze IE0, IE0^{MtoA} or IE1 and AC146HA expression Western blot analysis was performed. Cells were analyzed at 48 hpt and as expected, wildtype virus produced both IE0 and IE1 indicated at 72.6 and 66.9 kDa respectively (Fig. 10). Cells transfected with AcBac$^{ac146\text{-}ie1KO}$ showed no expression of IE0 and IE1, confirming the deletion of *ie1* (Fig. 10). AC146 was not expected to be detected in wildtype virus as it is not HA-tagged in its native form. As shown in Fig. 10 all viruses produced the expected protein products. The viruses expressing *ie0* under control of the *ie1* promoter or *gp64* promoter produced both IE0 and IE1. Whereas the viruses expressing *ie0*^{MtoA} only produced IE0 and the viruses expressing *ie1* only produced IE1.

Western blot analysis of AC146HA in viruses repaired with *ac146HA* all showed the presence of a 23 kDa protein, the predicted size of AC146 (Dickison et al., 2012). This showed that AcBac$^{ac146\text{-}ie1\text{KO}}$ was repaired with *ac146HA* under control of a heterologous promoter and polyA signal. The non-HA tagged *ac146* viruses were repaired in the same manner and all viruses produced IE0 and IE1, IE0 or IE1 as well as BV, indicating that *ac146* was successfully repaired.

3.5 Viral titre analysis

BV stocks were generated from bacmid transfected cells harvested at 9 dpt. Virus titres were determined and no difference was seen between the HA tagged *ac146* viruses and untagged viruses. Therefore, for the purpose of examining viruses expressing IE0, IE0^{MtoA} and IE1 under control of identical promoters, the wildtype virus and the six recombinant viruses expressing native, untagged AC146; v*ie1*p-IE0, v*ie1*p-IE0^{MtoA}, v*ie1*p-IE1, v*gp64*p-IE0, v*gp64*p-IE0^{MtoA}, v*gp64*p-IE1 were used for further experiments.

3.6 Virus time course assays

Time course assays with v*ie1*p-IE0, v*ie1*p-IE0^{MtoA}, v*ie1*p-IE1, v*gp64*p-IE0, v*gp64*p-IE0^{MtoA}, and v*gp64*p-IE1 were performed to compare DNA replication and BV production of viruses expressing *ie0*, *ie0*^{MtoA} or *ie1* under control of identical promoters. *Sf*9 cells were infected at an MOI of 5 and cell pellets and supernatants were collected at various time points for analysis.

3.6.1 IE0, IE0^{MtoA} and IE1 analysis by Western blot

The use of identical promoters to drive expression of *ie0*, *ie0*^{MtoA} or *ie1* was an attempt to obtain similar temporal expression and similar levels of expression of IE0, IE0^{MtoA} and IE1. It was therefore important to examine the level of IE0 and IE1 expression around the time of initiation of viral DNA replication (6-8 hpi), to ensure that any differences observed in viral DNA replication level were due to protein function rather than the level of protein expression.

Infected cell pellets harvested at 6, 7, 9, and 11 hpi were analyzed by Western blot (Fig. 11). IE0, IE0^{MtoA} and IE1 expression was detected using a monoclonal antibody that recognizes an epitope in the AAD present in IE0, IE0^{MtoA} and IE1. At 6 hpi all viruses produced nearly undetectable levels of protein but analysis of subsequent time points (7, 9 and 11 hpi), indicated that protein levels increased to detectable levels. At all time points examined viruses using the *ie1* promoter, (v*ie1*p-IE0, v*ie1*p-IE0^{MtoA}, and v*ie1*p-IE1) all expressed greater amounts of protein produced compared to viruses using the *gp64* promoter, (v*gp64*p-IE0, v*gp64*p-IE0^{MtoA}, and v*gp64*p-IE1) (Fig. 11). For each promoter, at each time point, the level of protein for IE0, IE0^{MtoA} and IE1 was comparable (Fig. 11). This allowed comparison of the function of IE0, IE0^{MtoA} and IE1 in viral DNA replication between the viruses containing the *ie1* or *gp64* promoter because the levels of protein expression were similar for each virus set.

The level of IE0, IE0^{MtoA} and IE1 expression was also analyzed throughout the virus replication cycle to examine temporal expression of IE0, IE0^{MtoA} and IE1 under control

of the *ie1* promoter or *gp64* promoter (Fig. 12). Infected cell pellets harvested at 6, 9, 12, 20, 24, 36 and 48 hpi were analyzed by Western blot. The expression of *ie0*^{MtoA} or *ie1* under control of the *gp64* promoter exhibited the same general temporal expression. That is, a steady increase of IE0^{MtoA} or IE1 expression up to 24 hpi followed by a large increase at 36 hpi and maintained to 48 hpi. For virus expressing *ie0* which produces mainly IE0 with a smaller amount of IE1, the large increase in steady state expression levels was detected between 12 and 20 hpi. Under control of the *ie1* promoter, *ie0*^{MtoA} and *ie1* exhibit the same profile with a continuous increase in expression to 48 hpi without large increases between 24 and 36 hpi, as seen when driven by the *gp64* promoter. However *ie0* driven by the *ie1* promoter shows the same temporal pattern as *gp64* driven *ie1* with a large increase in expression levels between 12 and 20 hpi.

3.6.2 DNA replication analysis by qPCR

Analysis of DNA replication was performed to compare viral replication of v*ie1*p-IE0, v*ie1*p-IE0^{MtoA}, v*ie1*p-IE1, v*gp64*p-IE0, v*gp64*p-IE0^{MtoA}, v*gp64*p-IE1 and WT. The amount of DNA replication for each virus at each time point was determined using qPCR to detect the number of genomes present in the harvested cell pellet. In WT infected cells, viral DNA replication initiates at approximately 6-8 hpi. Therefore time course data for each virus was normalized to 3 hpi, to reflect the increase in the number of genomes present due to DNA replication and viruses containing identical promoters driving *ie0*, *ie0*^{MtoA} and *ie1* expression were compared to each other (Fig. 11).

Viruses utilizing the *ie1* promoter to express IE0, IE0^{MtoA} and IE1 achieved similar DNA replication levels as wildtype virus. However differences were observed in the initiation of DNA replication. The viruses v*ie1*p-IE0^{MtoA}, and v*ie1*p-IE1 initiated DNA replication at similar times between 6-8 hpi. However, v*ie1*p-IE0 showed a 1-2 hour delay in initiation of DNA replication (Fig. 13A). This suggested expression of IE0 with small amounts of IE1 delays initiation of viral DNA replication when under control of the *ie1* promoter, compared to viruses that express IE0^{MtoA} or IE1. Even though DNA replication initiation times were dissimilar, all viruses showed an increase of viral DNA levels of over a thousand fold by 48 hpi which was similar to wildtype virus (Fig. 13A).

Viruses utilizing the *gp64* promoter express IE0, IE0^{MtoA} and IE1 at lower levels than the viruses utilizing the *ie1* promoter. When under control of the *gp64* promoter, expression of IE0 resulted in a virus that was most similar to wildtype virus with no delay in initiation of DNA replication and reached similar levels of DNA replication by 48 hpi (Fig. 13B). Whereas IE0^{MtoA} and IE1 both had delayed initiation of DNA replication (Fig. 13B) and the amount of DNA replication was lower and did not reach WT levels by 48 hpi. By 48 hpi, the expression of IE0 resulted in over a thousand fold increase in level of viral DNA, which differed from IE0^{MtoA} and IE1 which reached only a five hundred fold increase in level of viral DNA. The results show that when expression levels are lower due to the weaker *gp64* promoter, IE0 initiated DNA replication earlier and reached higher levels of viral DNA replication than either IE0^{MtoA} or IE1.

3.6.3 Temporal analysis of budded virus production

BV production was also examined in cells infected with IE0, IE0^{MtoA} and IE1 under control of the *ie1* and *gp64* promoters. Budded virus production was analyzed using qPCR for quantification of the number of genomes present in supernatants harvested at each time point for each virus. All data was normalized to the first time point at 3 hpi. As with the DNA replication analysis, viruses expressing IE0, IE0^{MtoA} and IE1 utilizing identical promoters were compared to each other.

Viruses utilizing the *ie1* promoter to express IE0, IE0^{MtoA} and IE1 all initiated BV production at 18 hpi and BV levels increased to about one thousand-fold by 48 hpi (Fig. 14A). These viruses, all expressing similar levels of IE0, IE0^{MtoA} or IE1, acted similarly to wildtype virus, except that wildtype virus reached slightly higher levels of BV production by 48 hpi.

Viruses utilizing the *gp64* promoter to express IE0, IE0^{MtoA} and IE1 showed a different BV production profile compared to wildtype. Expression of IE0 resulted in initiation of BV production at 18 hpi, like wildtype, and levels increased over one thousand-fold by 48 hpi, although levels were lower than wildtype (Fig. 14B). Expression of IE0^{MtoA} and IE1 show a very different BV profile as BV production was delayed, initiating between 20-24 hpi and BV levels increased only one hundred fold by 48 hpi (Fig. 14B). This result shows that at low levels of IE0 expression, which also produces small amounts of IE1, BV production initiates at least 6 hours before viruses expressing low levels of only

IE0^{MtoA} or IE1 (Fig. 14B). No difference was observed between IE0^{MtoA} and IE1 when expression levels were low.

3.7 Transactivation analysis of early gene promoters by IE0 and IE1

IE0 and IE1 have shown to be transactivators of viral early gene promoters (Guarino and Summers, 1986a) and an acidic activation domain has been identified (Pathakamuri and Theilmann, 2002; Taggart et al., 2012). Part of IE0 and IE1`s essential role in viral replication may be due to their ability to transactivate viral genes required for viral replication. Results of a previous study has shown that IE0 and IE1 activate the same viral early gene promoters, that is, no viral promoters were identified to be specifically transactivated by IE0 or IE1 (Nie, 2010). Differences were only observed in the levels of reporter gene transactivation and these differences could be simply due to variable levels of IE0 or IE1 which were expressed using different promoters. Therefore it was hypothesized that IE0 and IE1 are equal in ability to transcriptionally transactivate viral early gene promoters. To test this hypothesis, the ability of IE0, IE0^{MtoA} and IE1 to transiently transactivate viral early gene promoters when they were expressed utilizing the same promoter was examined. Nineteen early gene promoter-CAT reporter gene constructs were analyzed (Fig. 7). The reporter plasmid constructs were transfected by themselves or with plasmids expressing IE0, IE0^{MtoA} or IE1 under control of the *ie1* promoter or *gp64* promoter (Fig 7). CAT assays were performed to determine the level of transactivation of each viral early gene promoter by IE0, IE0^{MtoA} and IE1 at similar levels of expression. The data is presented

normalized to IE1 transactivation (Fig. 15A and 16A) or as counts per minute (Fig. 15B and 16B). Statistical analysis is summarized in Table 3 and 4.

3.7.1 Analysis of level of transactivation by IE0, IE0^{MtoA} and IE1

Of the nineteen viral early gene promoters examined, there was a wide range of response to the transactivators IE0, IE0^{MtoA} and IE1. Similar to what was previously reported, any early gene promoter that was transactivated by IE0^{MtoA} was also transactivated by IE1. Nine promoters appeared to be differentially expressed depending on which transactivator, or whether the transactivator was expressed at high (*ie1* promoter) or low (gp64 promoter) levels. At low expression levels eight promoters, *p35*, *orf18*, *lef4*, *39K*, *lef3*, *lef6*, *orf79* and *orf111* were transactivated to higher levels by IE0 and IE0^{MtoA} compared to IE1 (Fig. 16A). At high levels however, no difference was observed in the expression with *p35*, *orf18*, *lef4*, *lef3*, *lef6*. These promoters therefore appear to be more sensitive to IE0 when cellular levels are low. Expression of high levels of IE0, IE0^{MtoA} and IE1 revealed three viral early gene promoters, *orf111*, *ie1* and *39K* that were transactivated to a higher levels by IE1 compared to IE0 and IE0^{MtoA} (Fig. 15A). These data show that there appears to be specificity in promoter activation by IE0 and IE1 and it appears to be dependent on cellular levels of IE0, IE0^{MtoA} or IE1. Statistical analysis support these results (Table 3 and 4).Three viral early gene promoters *ie0*, *me53*, and *gp64* had relatively high levels of expression in the absence of transactivator and showed no increase or decrease when co-transfected with plasmids expressing IE0, IE0^{MtoA}, or IE1. These results show that these promoters were not transactivated by any transactivator but were actively expressed by cellular

transcription factors (Fig. 15B and 16B). The reporter constructs containing the promoters of *orf33*, *orf91*, *orf52* and *orf76* resulted in CAT expression levels that were barely above background and only minor or no increase was observed when transactivated by IE0, IE0$^{Mto\ A,}$ or IE1. These viral promoters are therefore not activated by cellular proteins and the increase in CAT expression that is observed would be consistent with the ability of IE0 or IE1 to non-specifically activate basal promoters at low levels (Choi and Guarino, 1995c) (Fig. 15B and 16B). Down regulation of the *pe38* promoter was detected when IE1 was expressed from the *gp64* promoter though decrease of expression was minimal. IE0^{MtoA} when expressed from either the *ie1*or *gp64* promoters had a similar impact on the *pe38* promoter but no impact, positive or negative, was seen on the *ie2* promoter (Fig. 16 A and B). The *p78* promoter appears to be insensitive to low level expression (*gp64* promoter) of either IE0 or IE1 but showed activation at higher levels (*ie1* promoter) (Fig. 15 and 16). For the *p78* promoter therefore, there appears to be a threshold level of transactivator required but no differences were observed between IE0 or IE1.

3.7.2 IE0, IE0^{MtoA} and IE1 analysis by Western blot

Western blots were performed to compare the levels of expression of IE0 and IE1 in the transient expression assays. In general, IE0, IE0^{MtoA} and IE1 under control of the *gp64* promoter resulted in lower expression levels than when under control of the *ie1* promoter (Fig. 17). The levels of IE0^{MtoA} and IE1 produced under control of the *gp64* promoter were relatively low but similar. The expression of IE0 produced a slightly greater amount of IE0/IE1 from internal translation initiation (Fig. 17). Under control of

the *ie1* promoter, the level of IE0 and IE0^{MtoA} were similar but IE1 showed greater levels. This would suggest that IE1 autoregulates the *ie1* promoter which agrees with the CAT assay results (Fig. 15A) that showed that IE1 transactivated the *ie1* promoter to higher levels than IE0 or IE0^{MtoA}. The interpretation of the CAT assay results using the *ie1* promoter to drive expression of IE0, IE0^{MtoA} and IE1 has to take into account the higher expression of IE1. The differences in the transactivation of early gene promoters by IE0^{MtoA} or IE1 were therefore more readily apparent at the low equal levels that is observed using the *gp64* promoter.

However using the *ie1* promoter, the transactivation levels of IE0, IE0^{MtoA} and IE1 were all higher and very few differences in transactivation were observed, even though IE1 was expressed at higher levels. This would suggest that transactivation of the early gene promoter-*cat* reporters were being maximized by high levels of IE0, IE0^{MtoA} and IE1 expression, masking any functional differences between IE0^{MtoA} and IE1. Exceptions were observed, the *39K* and *orf111* promoters were transactivated to greater levels by IE0 and IE0^{MtoA} when at low levels of protein expression (*gp64* promoter, Fig. 16A) but transactivated to higher levels by IE1 when at high levels of protein expression (*ie1* promoter, Fig. 15A).

The CAT assay results would suggest that promoters are differentially sensitive to transactivation by IE0, IE0^{MtoA} and IE1 depending on the intracellular levels. This is theoretically described in Fig. 18A which shows that at low levels of expression certain promoters are more sensitive to IE0 and IE0^{MtoA} than IE1 and are activated at low cellular levels. As the levels of transactivator increase the promoters become more

responsive to IE1 as they reach maximum expression levels. Once maximum levels are reached, the differences between IE0, IE0^{MtoA} and IE1 are no longer observed. To test this model of the functional differences between IE0, IE0^{MtoA} and IE1, preliminary experiments with increasing amounts of transactivator and monitoring the level of transactivation were initiated (Fig. 18B). It was expected that as the amounts of IE0^{MtoA} and IE1 increased, rate of transactivation would also increase, until a maximum rate was achieved. It was also expected that the rate of transactivation observed by IE0^{MtoA} would be higher than IE1, when amounts of transactivator were low (*gp64* promoter, Fig. 18A). However, when amounts of transactivators increased to higher levels (*ie1* promoter, Fig. 18A), it was expected that the rate of transactivation by IE1 would surpass rates achieved by IE0 and IE0^{MtoA}, until all transactivators achieved the maximum transactivation rate.

The preliminary experiment analyzed the level of transactivation of the *39K-cat* reporter plasmid using a range of 0.5 to 2.5 µg of transfected plasmid expressing IE0, IE0^{MtoA} or IE1 under control of the *gp64* promoter (Fig. 18B). As the amount of transfected IE0 or IE0^{MtoA} expressing plasmid increased, the level of transactivation increased, though the levels of transactivation achieved with IE0 were higher than IE0^{MtoA} (Fig. 18B). However, as the amount of transfected plasmid expressing IE1 increased, no increase in the levels of transactivation was observed (Fig. 18B). These results would agree with the theoretical results in Fig. 18A but further assays with higher levels of transactivator are required. However, this result confirms that the *39K* promoter is more sensitive to

IE0 and IE0^{MtoA} transactivation than IE1 transactivation showing that viral promoters are differentially transactivated by IE0 and IE1.

4 Discussion

The goal of this study was to compare the function of AcMNPV IE0 and IE1 in DNA replication and early gene transactivation when IE0, IE0^{MtoA} and IE1 were produced at similar cellular levels. Previous examination of the function of IE0^{MtoA} and IE1 was conducted under control of their native *ie0* and *ie1* promoters (Nie, 2010; Stewart et al., 2005). When IE0, IE0^{MtoA} or IE1 were placed under control of their native promoters, different amounts of IE0, IE0^{MtoA} or IE1 were obtained, and any functional difference observed may have been due to differences in level of expression (Nie, 2010). In this study therefore, a new approach was used and identical promoters were used to control *ie0*, *ie0^{MtoA}* or *ie1* to produce IE0 and IE1, IE0 or IE1 respectively. The results indicate that IE0^{MtoA} and IE1 are approximately equivalent in their ability to initiate and support viral DNA replication but show synergistic affects when expressed together. The two proteins however do appear to be functionally different in their ability to transactivate viral gene expression.

4.1 Comparison of IE0, IE0^{MtoA} and IE1 in time course assays

The initial hypothesis of this study was that IE1 was more efficient in DNA replication compared to IE0^{MtoA} which was based on previous research that showed virus producing only IE1 followed DNA replication profiles similar to wildtype virus (Stewart et al., 2005). In this study, time course assays revealed differences in initiation and level of DNA replication and budded virus production for viruses expressing IE0, IE0^{MtoA} or IE1. Expression of IE0^{MtoA} and IE1at high or low levels (Fig. 13) showed that these two proteins supported virus replication and that initiation and final levels were

approximately equal. However, if IE0^{MtoA} or IE1 were expressed at sustained lower levels (*gp64* promoter) and compared to cells infected with WT virus, initiation of viral DNA replication was delayed and final levels were lower. When expressed at higher levels (*ie1* promoter) IE0^{MtoA} or IE1 were very similar to WT. The surprising result was that low level expression of IE0 (Fig. 13B) resulted in viral DNA replication initiation and final levels nearly identical to WT. Expression of *ie0* results in both IE0 and IE1 being produced (Fig. 12) so this clearly shows that there is a synergistic interaction between these two proteins that results in enhanced viral DNA replication compared to either protein by itself. However, expression of *ie0* at higher cellular levels (Fig. 13A) results in an antagonistic interaction and viral DNA initiation is delayed but levels eventually reach WT levels. Antagonistic effects between IE0 and IE1 have been shown in regards to viral late gene expression (Huijskens et al., 2004).

From the perspective of the virus infection, these results strongly suggest that there is a distinct advantage for expressing both IE0 and IE1 as it results in more potent stimulation of viral DNA replication compared to each protein separately. This result would provide an explanation of why the spliced *ie0* is translated as both proteins early in infection when levels are low (Theilmann et al., 2001) and why expression decreases late in infection.

DNA replication can have a major impact on the timing and level of BV production (Milks et al., 2003), the effect of IE0, IE0^{MtoA} and IE1 on BV production was examined. At relatively higher levels, (*ie1* promoter), no difference was seen between IE0, IE0^{MtoA} and IE1; all were similar in timing and level of BV production. At relatively lower levels, (*gp64*

promoter), IE0^{MtoA} and IE1 were equivalent in timing and level of BV production. The level of BV production of IE0^{MtoA} and IE1 increased by only a hundred fold and a BV initiation was delayed. However, IE0 initiated BV production at the same time as wildtype virus (18 hpi) and BV levels increased to approximately one thousand fold, which was slightly less than wildtype.

4.2 Comparision of IE0, IE0^{MtoA} and IE1 in transactivation of viral early gene promoters

Part of the initial hypothesis of this study was that IE0 and IE1 were equal in their ability to transactivate viral early gene promoters. This was based on a previous study that showed that all promoters that were transactivated by IE0 were also transactivated by IE1 (Nie, 2010) which indicated that there are no specific IE0 or IE1 activated promoters. However, it was possible that a promoter may be more sensitive to activation by IE0 or IE1. To address this question in this study, IE0 and IE1 were expressed using identical promoters to produce approximately equal levels of expression to determine if the levels of transactivation by IE0 and IE1 were the same or different.

Nineteen viral early gene promoters were used for this analysis. One set of viral early gene promoters were chosen because they have been reported as being transactivated by IE1 in the literature; *39K* (Guarino and Summers, 1986a), *p35* (Schultz et al., 2009), *gp64* (Blissard and Rohrmann, 1991b), *ie0* (Kovacs et al., 1991), *ie1* (Kovacs et al., 1991; Pullen and Friesen, 1995b), *pe38* (Leisy et al., 1997) and *ie2* (Leisy et al., 1997).

Whereas other viral early gene promoters that were used were recently identified to be potentially differentially regulated by IE0 and IE1 by microarray analysis; *orf18*, *lef4*, *lef3*, *lef6*, *orf79*, *orf111*, *orf91*, *orf52* and *orf76* (Nie, 2010). Four viral early gene promoters, *orf33*, *orf91*, *orf52* and *orf76* that had previously been suggested to have differential activation by IE0, IE0^{MtoA} or IE1 by microarray studies (Nie, 2010), showed no activation in this study. This would therefore suggest that additional viral factors are required for transactivation of these promoters. Down regulation of the *pe38* promoter was seen when IE1 was expressed from the *gp64* promoter. The *ie2* and *pe38* promoters have been previously reported to be down regulated by IE1 (Leisy et al., 1997).

IE0, IE0^{MtoA} and IE1 produced at low levels (*gp64* promoter) and high levels (*ie1* promoter) were analyzed for their ability to transactivate each of the nineteen viral early gene promoters. When IE0, IE0^{MtoA} and IE1 were at low levels, differences in transactivator function were readily observed. Both IE0 and IE0^{MtoA} transactivated eight viral early gene promoters *(lef4, lef6, orf79, p35, orf18, lef3, orf111,* and *39K*) to a higher level than IE1 (Fig. 16A). Statistical analysis generally support these results ($p<0.05$, Table 3). One statistical exception is *lef4* where IE1 compared to IE0^{MtoA} $p=0.13$, however IE1 compared to IE0 $p=0.002$. A subset of four promoters, *lef4, lef6, orf79* and *39K*, were transactivated to a higher level by IE0 compared to IE0^{MtoA}. Statistical analysis support these results ($p<0.05$, Table 3), with two exceptions. The p values for IE0 compared to IE0^{MtoA} for *lef4* was 0.08 and *orf79* was 0.25. This shows that IE0 and IE0^{MtoA} are more potent transactivators of specific promoters than IE1

when present at low levels. This result would provide an explanation of why IE0 is present in higher amounts relative to IE1 at early times in infection (Huijskens et al., 2004).

A recent study has also compared the function of IE0^{MtoA} and IE1 examining both transcriptional activation and DNA replication (Luria et al., 2012). Transcriptional transactivation was analyzed using a luciferase reporter gene under control of the *p39K* promoter that was linked to an *hr* enhancer. IE0^{MtoA} and IE1 were expressed under control of the *Drosophila melanogaster* hsp70 promoter (Luria et al., 2012). Under these *hr* dependent conditions transactivation by IE0^{MtoA} was 90-fold greater than IE1 (Luria et al., 2012). This result would agree with the results of this study showing IE0^{MtoA} was a stronger activator of *p39K* promoter however, transactivation by IE0 was not explored (which produces small amounts of IE1) or *hr*-independent transactivation. *Hr*-dependent transactivation depends on direct binding of IE0 or IE1 to the *hr* element (Guarino and Dong, 1991; Kovacs et al., 1992; Rodems and Friesen, 1993) whereas in this study, *hr*-independent transactivation without direct binding was examined. In addition, evidence showing that that similar IE0^{MtoA} and IE1 protein expression levels were achieved was not provided.

When expressed at higher levels (*ie1* promoter) the differences between IE0, IE0^{MtoA} and IE1 became less apparent. IE1 transactivated three viral early gene promoters, *orf111, 39K* and *ie1*, to a higher level than IE0 and IE0^{MtoA} (Fig. 15B, Table 4 for statistical analysis) but this could be due to high levels of expression observed. The six promoters *(lef4, lef6, orf79, p35, orf18* and *lef3*) that were more strongly activated by

IE0 and IE0^{MtoA} at low levels showed approximately equal transactivation with IE1 when expression levels were higher. This data suggests that maximum levels of transactivation and reporter gene expression may be occurring when each of the transactivators are produced at high levels (*ie1* promoter). Since transactivation may be at its maximum, this could prevent the detection of functional differences between IE0^{MtoA} and IE1.

Taken together, the results indicate that functional differences between IE0 and IE1 are dependent on cellular concentration. This is graphically described in Figure 18A which illustrates a theoretical example of how transactivators may behave according to the observations seen in this study when present at high and low levels. Specific promoters appear to be more sensitive to IE0 and IE0^{MtoA} so that at low levels of expression the level of transactivation is greater than IE1. In addition, levels of transactivation achieved with IE0 are higher than levels of transactivation achieved with IE0^{MtoA}. When IE1 reaches high enough levels the ability to transactivate starts to equal that of IE0 and IE0^{MtoA}. Once a promoter reaches maximal level of expression no differences will be observed between IE0, IE0^{MtoA} or IE1. The results in general appear to support this model. Viral early gene promoters that show equivalent transactivation by all transactivators at high levels (*lef4, lef6, orf79, p35, orf18,* and *lef3*) could represent the situation where rate of transactivation is maximized, when transactivator amounts are high (top right of graph). However, these same viral early gene promoters show increased transactivation by IE0 and IE0^{MtoA} compared to IE1 when the transactivators are at low levels (lower left of graph). The promoters *orf111* and *39K* fit the model as

well; when the transactivators were present at low levels, there was increased activation by IE0 and IE0^{MtoA} compared to IE1 (lower left of graph) but at high levels there was increased activation by IE1 compared to IE0 and IE0^{MtoA} (upper right of graph). The *ie1* promoter appears to be an exception as it showed no transactivation by any of the transactivators at low levels but activation by only IE1 was observed at high levels. This suggests the *ie1* promoter is specifically activated by IE1 but unlike the other promoters requires higher levels of expression for transactivation. The Western blot of transient transfection also supports this observation as p*ie1*-IE1 expressed higher levels of transactivator than either p*ie1*- IE0 or p*ie1*-IE0^{MtoA} indicating autoregulation by IE1 (Fig. 17). The remaining promoters that showed no activation compared to the reporter plasmid alone therefore are not responsive to IE0, IE0^{MtoA} or IE1. However, it is possible that in the context of other viral factors during viral infection IE0, IE0^{MtoA} or IE1 may play a role in their expression.

4.3 Significance of findings in context of virus infection

IE0 and IE1 are key transregulatory proteins, either IE0 or IE1 are needed to support viral replication but both are required for wildtype replication (Stewart et al., 2005), suggesting that each protein has a specific role in the viral replication cycle. Of the four genera of *Baculoviruses*, *Alphabaculoviruses* are the most recent evolutionary group (Krell, 2008) and are the only genera that utilize gene splicing which only produces *ie0* (Chisholm and Henner, 1988). For *Alphabaculoviruses* to employ this relatively complex mechanism to produce IE0 and IE1, it is likely that these proteins have a distinct advantage for the virus.

During the first few hours of infection of an insect, cellular levels of all viral proteins including the immediate early proteins like IE0 and IE1 are low. There is evidence that IE1 is packaged in ODV indicating thatIE0 and IE1 may be present in the cell at low levels during initial nucleocapsid entry (Braunagel et al., 2003). The study by Braunagel et al. (2003) identified IE1 in AcMNPV ODV although this conflicted with published results in OpMNPV which showed IE1 only associating with BV rather than ODV (Theilmann and Stewart, 1993). This difference may have been due to differences in protein preparation and suggested that IE1 may be easily susceptible to degradation (Braunagel et al., 2003). Therefore, if in AcMNPV ODV low levels of IE1 are present and low abundance IE0 may be present, but may not have been detected in their analysis. Sustained low levels expression of IE0, IE0^{MtoA} and IE1 using the *gp64* promoter highlights the early events that may be occurring in WT infected cells. Regardless of whether IE0 and IE1 are packaged within ODV, the number of initial nucleocapsids that enter the midgut cells may be small. Therefore, at these early times in infection within the midgut cells, production of immediate early proteins like IE0 and IE1 may be relatively low. The results obtained from viruses using the *gp64* promoter, when low levels of IE0 and IE1 are produced, may be used to speculate how IE0 and IE1 function at these early times in infection. After the entry into the midgut cell and production of viral proteins accumulates, levels of immediate early proteins are increased (Huijskens et al., 2004). These later times, when higher levels of IE0 and IE1 are present, may be represented in this study by the viruses using the *ie1* promoter, which expressed higher levels of IE0 and IE1.

At early stages of viral infection the insect employs defence mechanisms to rid itself of the virus that result in midgut cell death (reviewed in Hakim et al., 2010). The virus must compete with the cell's defence mechanisms in order to replicate and produce a systemic infection. One insect defence mechanism is sloughing, where the infected midgut cell shed and it swells and bursts followed by new midgut cell proliferation (reviewed in Hakim et al., 2010). Sloughing is a defence mechanism used for baculovirus infection resulting in prevention or delay in virus infection (Hoover et al., 2000; reviewed in Clem, 2005). Therefore it is important that the virus rapidly produces BV for systemic infection or else the virus infected cell is sloughed and the virus can no longer replicate. At these early stages of infection when viral cellular proteins are at low levels, the virus must find a mechanism to rapidly move out of the midgut and into other cell types. One strategy may be the stimulation of rapid viral DNA replication by both IE0 and IE1 together, resulting in BV production and subsequent systemic infection. Therefore co-production of both IE0 and IE1 proteins, ensured by internal translation (Theilmann et al., 2001) is advantageous to evade the hosts defence strategy of sloughing to ensure successful infection.

Another insect defence strategy is programmed cell death, known as apoptosis which is conserved among most animals (reviewed in Rohrmann, 2011). In insects, apoptosis is an antiviral defence mechanism, and serves many other purposes including preventing unwanted cell division, removal of extraneous tissue during organism development as well as damaged cells (reviewed in Clarke and Clem, 2003). Infected cells are triggered into apoptosis by either viral DNA replication or expression of a viral gene at the same

time as viral DNA replication (Schultz and Friesen, 2009). It is important for the virus to have viable host cells and accompanying cellular machinery in order for the virus to replicate. Therefore viruses have evolved to include mechanisms to regulate or inhibit apoptosis, to counteract the host's defence mechanism of apoptosis. AcMNPV produces the protein P35, which prevents apoptosis in infected cells (Clem et al., 1991; Clem and Miller, 1993; Lee et al., 1998; reviewed in Clem, 2005). Part of the apoptosis process is the use of initiator and effector caspases to disassemble the cell (reviewed in Clem, 2005). Initiator caspases activate other caspases and effector caspases cleave cellular components. P35 binds to effector caspases, which results in cleavage of P35, but also causes a conformation change in the effector caspase that permanently binds the proteins together, preventing apoptotic cell death (reviewed in Clarke and Clem, 2003).

The *p35* viral early gene promoter was one of the promoters identified as transactivated to a higher level by IE0 or IE0^{MtoA} compared to IE1. Again, if low levels represent early times in virus infection, when evasion of the host's defence system is critical, increased cellular P35 would be advantageous for apoptosis prevention, and may promote subsequent viral replication. Currently, it has not been shown that apoptosis occurs in insect midgut cells. However, apoptosis occurs in other cells types that are affected by systemic infection (Clarke and Clem, 2003). In these cell types, presence of IE0 may produce increased levels of P35, providing a mechanism against apoptosis resulting in successful infection.

Another viral early gene promoter identified as activated to a higher level by IE0 or IE0^{MtoA} compared to IE1 was *lef3*. LEF3 has been widely studied and has been found to be critical for viral DNA replication (Nie et al., 2012), as well as required for transient DNA replication (Kool et al., 1994a; Lu and Miller, 1995). LEF3 is a single stranded binding protein (Hang et al., 1995), which stabilizes the unwound DNA at the replication fork during DNA replication (Kool et al., 1995). An increase in DNA replication factors, including LEF3 is advantageous for increased DNA replication, especially at early times in virus infection. Other DNA replication factors, HELICASE, DNA POL, LEF1 and LEF2 were not analyzed for transactivation by IE0, IE0^{MtoA} or IE1, as they were not previously identified by microarray or other analyses. It has been shown that DNA replication affects BV production (Milks et al., 2003) and an increase in BV may increase the opportunity for systemic infection. However, it should be noted that even though at low transactivator levels (*gp64* promoter), IE0^{MtoA} showed higher transactivation of the *lef3* promoter compared to IE1, the virus time course assays showed no difference in initiation or levels of DNA replication with viruses expressing IE0^{MtoA} and IE1 at low levels (*gp64* promoter). Therefore, increased transactivation by IE0^{MtoA} compared to IE1 of any of the viral early gene promoters studied does not result in an increase in viral DNA replication. It is the combination of IE0 and IE1 that results in early initiation of viral DNA replication and higher viral DNA levels. It is possible that increased transactivation by IE0^{MtoA} combined with a more efficient replication complex containing IE0 and IE1 allows rapid viral DNA replication and successful systemic infection.

It is tempting to associate all viral early gene promoters that were differentially regulated by IE0 or IE0^{MtoA} with processes important to rapid viral replication like apoptosis prevention. Unfortunately, the remaining viral early gene promoters produce proteins that function in a wide variety of viral processes, and most are not thoroughly characterized. Functions for both ORF18 and ORF111 are currently unknown (reviewed in Rohrmann, 2011). LEF6 and 39K are involved in late gene expression (Todd et al., 1995). However 39K has also been shown to bind DNA (Guarino et al., 2002) as well as be transactivated by IE1 (Guarino and Summers, 1986a). ORF79 has been suggested to be involved in DNA repair (reviewed in Rohrmann, 2011). LEF4 has been found to be an RNA 5' capping enzyme (Gross and Shuman, 1998; Jin et al., 1998), which serves to protect from degradation and interact with translation initiation factors. These proteins are involved in processes that are important to the virus and further studies are needed to fully understand their roles and interactions with IE0 and IE1.

4.4 Conclusions

This study's aim was to determine the functional differences of IE0 and IE1 in DNA replication and transactivation of viral early gene promoters. Differences between these transregulatory proteins was more readily seen when they were expressed at low levels. This study shows that both IE0 and small amounts of IE1 are required for efficient DNA replication. However IE0 appears to have enhanced ability to transactivate a set of viral early gene promoters compared to IE1.

Considering that *Alphabaculoviruses* are the only genera to contain *ie0*, the only known baculovirus spliced gene that produces multiple protein products, an attractive idea is that IE0 and IE1 play a role in viral replication that make this group of viruses robust. Efficient DNA replication by the presence of both IE0 and IE1 and enhanced transactivation of some viral early gene promoters by IE0 may benefit the virus when viral protein levels are low. This supports IE0 and IE1's role as proteins that confer a distinct advantage to the virus.

4.5 Future studies

The results of this study lead to more questions regarding IE0 and IE1's roles in AcMNPV replication. Additional studies involving DNA replication and early gene transactivation are needed to support the results from this study.

The finding that IE0 and IE0^{MtoA} preferentially transactivate some viral early gene promoters may be refined with a more thorough analysis. It was discussed that viral early gene promoters that are preferentially upregulated by IE0 and IE0^{MtoA} may accelerate events important to evade the insect's defence mechanisms and promote rapid virus replication and systemic infection. It is hypothesized that viral promoters associated with these events may also be upregulated, for example promoters for the essential DNA replication factors, *lef1*, *lef2*, *DNApol* and *helicase*. It is hypothesized that the genes for the remaining essential factors may be upregulated to contribute to rapid viral DNA replication, as shown in this study with essential viral replication factor LEF3. However, it would be expected that promoters not associated with rapid virus replication

or systemic infection would not be upregulated, for example *per os* infectivity factors that are required for infection of midgut cells (reviewed in Rohrmann, 2011).

To confirm the viral time course finding that IE0 and IE1 are required together for efficient viral DNA replication, further studies examining transient DNA replication are needed. Transient studies use only the viral replication factors, without any other viral factors present. Therefore these studies would truly show if IE0 and small amounts of IE1 were causing an increase in DNA replication, or if other viral factors are involved. Transient DNA replication studies use plasmids encoding all essential viral replication factors, a plasmid expressing IE0, IE0^{MtoA} or IE1 and a target plasmid including a viral origin of replication. The target plasmid containing the origin of replication is quantified, reflecting the abilities of IE0, IE0^{MtoA} or IE1 in DNA replication. It is expected that the level of target DNA replication achieved by transiently expressed IE0 when at low levels (*gp64* promoter) will be greater than IE0^{MtoA} and IE1 when present at low levels (*gp64* promoter). It is also expected that at high levels of IE0, IE0^{MtoA} or IE1 (*ie1* promoter), no difference in target DNA replication will be seen. This would confirm the findings in this study that at low levels, IE0 and IE1 are required together for rapid and efficient viral DNA replication.

To examine these findings in the context of insect infection, *in vivo* studies may be used. ODV expressing IE0, IE0^{MtoA} and IE1 under control of the *ie1* promoter and *gp64* promoter were collected when budded virus was produced during this study. The number of ODV could be quantified and fed to insects. It would be expected that the insects that were fed low amounts of viruses expressing IE0 at sustained low levels

(*gp64* promoter) would have a lower lethal dose (LD50) than viruses expressing low levels of IE1 or $IE0^{MtoA}$.

Table 1: Manufactured kits used

Kit Name	Use	Distributor
Qiaprep Spin Miniprep Kit	Plasmid purification – small scale	Qiagen
QIAfilter™ Plasmid Midi Kit	Plasmid purification – medium scale	Qiagen
Qiaquick Gel Extraction Kit	Gel extraction	Qiagen
EZ-10 Spin Column Plasmid DNA Miniprep Kit	Plasmid purification – small scale	VWR
EZ-10 Spin Column DNA Gel Extraction	Gel extraction	VWR
EZNA Fastfilter® Plasmid Midi Kit	Plasmid purification – medium scale	VWR

Table 2: Complete list of primer sequences and features

All primers were originally designed by N. Sokal unless otherwise indicated.

Primer number	Primer sequence 5' to 3'	Description of use and unique features
1551	acggctcgcatgtatagaaactt gttactatg*aaataaa*ggatctct gcagcacgtgtt	- used to construct the zeocin cassette for homologous recombination into wildtype bacmid, to knockout *ac146* and *ie1* loci - flanking 5' region of *ac145*, a polyA signal (italics) used for *ac145* - and a region homologous to the EM7 promoter-*zeocin* cassette (underlined) - previously designed (Dickison et al., 2012)
1918	aaaaataatataaaatatatgtat aattaattaaattca*aaataaa*g gcggtgttggtcggcgtcgg	- used to construct the zeocin cassette for homologous recombination into wildtype bacmid, to knockout *ac146* and *ie1* loci - flanking 3' region of *ie1* and *pif5*, a polyA signal used for *pif5* (italics) - a region homologous to the EM7 promoter-*zeocin* cassette (underlined)
1574	cgccagctgcaagcctatc	- used to confirm recombination 5' end of zeocin cassette - previously designed (Dickison et al., 2012)
520	ccggaacggcactggtcaactt	- used to confirm recombination 5' end of zeocin cassette - previously designed (Fang et al., 2007)
1919	gtccacgaacttccgggacgc	- used to confirm recombination 3' end of zeocin cassette
1920	ctatgcaaaaccccacaccaa	- used to confirm recombination 3' end of zeocin cassette
1936	aacctgcag*ttgtttcatattaagc cacaacgccgcccaac*atgaa cgtcaatttatactg	- used to amplify *ac146* from AcMNPV strain E2 - contains a *PstI* restriction site (underlined) - contains the OpMNPV *op146* promoter (italics)

Primer number	Primer sequence 5' to 3'	Description of use and unique features
1935	tta<u>ctcgag</u>*ttttataaaatttatta aaa*ctatgaagagcgggtttcca	- used to amplify *ac146* from AcMNPV strain E2 - contains a *XhoI* restriction site (underlined) and the OpMNPV *ie2* polyA signal (italics)
1940	tta<u>ctcgag</u>*ttttataaaatttatta aaa*ctatga**ggcgtagtcggg cacgtcgta**ggggtaagagcg ggtttcca	- used to amplify *ac146* from AcMNPV strain E2 and insert HA tag - contains a *XhoI* restriction site (underlined), the OpMNPV *ie2* polyA signal (italics) and sequence coding for the HA-epitope tag (bold)
1932	tcc<u>gaattc</u>*tctaga*gagtcgatg tctttgtgatgc	- used to amplify 550bp of IE1 promoter at 5' end - contains *EcoRI* (underlined) and *XbaI* (italics) restriction sites
1931	agt<u>ggatcc</u>cacttggttgttcac gatc	- used to amplify 550bp of IE1 promoter at 3' end - contains *BamHI* (underlined) restriction sites
1933	ccg<u>gaattc</u>*tctaga*aactgccg ttgctaagaaa	- used to amplify 350 p of the GP64 promoter at 5' end - contains *EcoRI* (underlined) and *XbaI* (italics) restriction sites
1934	cat<u>ggatcc</u>cttgcttgtgtgttcct tat	- used to amplify 350 bp of the GP64 promoter at 3' end - contains *BamHI* (underlined) restriction site
1937	taa<u>ctgcag</u>*ttttataaaatttatta aaa*ttaattaaattcgaatttttat at	- used to amplify *ie1*, *ie0* and $ie0^{MtoA}$ at 3' end - containing a *PstI* restriction site (underlined) and the OpMNPV *ie2* polyA signal (italics)
1938	aac<u>ggatcc</u>actatgacgcaaa ttaattttaa	- used to amplify *ie1* at 5' end - contains *BamHI* restriction site (underlined)
1939	aac<u>ggatcc</u>aacataagaacc agcagtc	- used to amplify *ie0* and $ie0^{MtoA}$ at 5' end - contains *BamHI* restriction site (underlined)

Table 3: Statistical p values comparing transactivation by IE0, IE0^{MtoA}, IE1 and reporter under control of the *gp64* promoter

	lef4	***lef6***	***orf79***	***p35***	***orf18***	***lef3***	***orf111***	***39K***
IE1-IE0^{MtoA}	0.1289758	0.0056196	0.0000991	0.0056848	0.1254680	0.0068448	0.0001710	0.0000311
IE1-IE0	0.0015394	0.0000667	0.0000096	0.0018176	0.0006965	0.0037661	0.0010138	0.0000001
IE0-IE0^{MtoA}	0.0824460	0.0433816	0.2493269	0.8865655	0.3050333	0.9817456	0.6026845	0.0001076
reporter-IE0	0.0060070	0.0000310	0.0000121	0.0004428	0.0000863	0.0014462	0.0009919	0.0000000
reporter - IE0^{MtoA}	0.0459157	0.0020559	0.0001293	0.0012721	0.0010983	0.0025612	0.0001677	0.0000018
reporter-IE1	0.9189258	0.9187394	0.9967098	0.7764441	0.4506629	0.9252972	0.9999988	0.7998730

	ie1	***ie2***	***pe38***	***me53***	***ie0***	***gp64***	***p78***	***orf33***
IE1-IE0^{MtoA}	0.9856646	0.3401793	0.9735156	0.0373980	0.9896275	0.9821896	0.9708943	0.9989782
IE1-IE0	0.1664496	0.6181200	0.9832343	0.2240093	0.9994087	0.2261122	0.0375722	0.1555688
IE0-IE0^{MtoA}	0.2741175	0.0509057	0.9999072	0.6852870	0.9737809	0.3734600	0.0774226	0.1252253
reporter-IE0	0.1872693	0.0440368	0.0704402	0.9346135	0.9762722	0.9790252	0.0045680	0.0596298
reporter - IE0^{MtoA}	0.9327010	0.9997480	0.0635663	0.9485345	0.9999988	0.5809769	0.3635831	0.9667070
reporter-IE1	0.9998199	0.3028811	0.1258321	0.0905602	0.9910040	0.3837544	0.5962397	0.9309352

	orf91	***orf52***	***orf76***
IE1-IE0^{MtoA}	0.7069298	0.7037707	0.0101844
IE1-IE0	0.7114728	0.0875193	0.0051693
IE0-IE0^{MtoA}	0.9999998	0.0143386	0.9747319
reporter-IE0	0.8838165	0.0023111	0.1493108
reporter - IE0^{MtoA}	0.8870553	0.6803535	0.2755635
reporter-IE1	0.3226023	0.1784549	0.2299548

Table 4: Statistical p values comparing transactivation by IE0, IE0^{MtoA}, IE1 and reporter under control of the *ie1* promoter

	lef4	***lef6***	***orf79***	***p35***	***orf18***	***lef3***	***orf111***	***39K***
IE1-IE0^{MtoA}	0.6044951	0.4748934	0.6433514	0.9606486	0.3316023	0.4312003	0.0004994	0.0000222
IE1-IE0	0.9925657	0.9998019	0.0799047	0.5685961	0.7146940	0.0307033	0.0006420	0.0006270
IE0-IE0^{MtoA}	0.4525415	0.5190647	0.7673590	0.8361563	0.8898289	0.3553500	0.9979909	0.0996424
reporter-IE0	0.0000000	0.0000024	0.0349171	0.0001624	0.0000173	0.0000333	0.0037799	0.0000000
reporter - IE0^{MtoA}	0.0000000	0.0000007	0.0068813	0.0000559	0.0000396	0.0000050	0.0050005	0.0000000
reporter-IE1	0.0000000	0.0000026	0.0004731	0.0000312	0.0000055	0.0000011	0.0000028	0.0000000

	ie1	***ie2***	***pe38***	***me53***	***ie0***	***gp64***	***p78***	***orf33***
IE1-IE0^{MtoA}	0.0102131	0.6619064	0.9899266	0.2421945	0.9817166	0.9207470	0.9995084	0.9911960
IE1-IE0	0.0066550	0.6300625	0.2407727	0.9973103	0.9446632	0.7363538	0.2582296	0.7909265
IE0-IE0^{MtoA}	0.6826013	0.9999361	0.1535032	0.3164208	0.7946923	0.9770718	0.2208427	0.8550573
reporter-IE0	0.6089236	0.9190729	0.0031622	0.0177456	0.7094098	0.6820329	0.0002017	0.0258803
reporter - IE0^{MtoA}	0.9992110	0.9365342	0.1407981	0.3182812	0.9985272	0.8851998	0.0000159	0.0069169
reporter-IE1	0.0082786	0.9352706	0.0870535	0.0128756	0.9504921	0.9996593	0.0000180	0.0055782

	orf91	***orf52***	***orf76***
IE1-IE0^{MtoA}	0.7995118	0.9973397	0.9961612
IE1-IE0	0.7963185	0.3698671	0.9750980
IE0-IE0^{MtoA}	0.9999999	0.4666797	0.9201508
reporter-IE0	0.2043950	0.0003899	0.0144515
reporter - IE0^{MtoA}	0.2062922	0.0000541	0.0051358
reporter-IE1	0.6343720	0.0000428	0.0073090

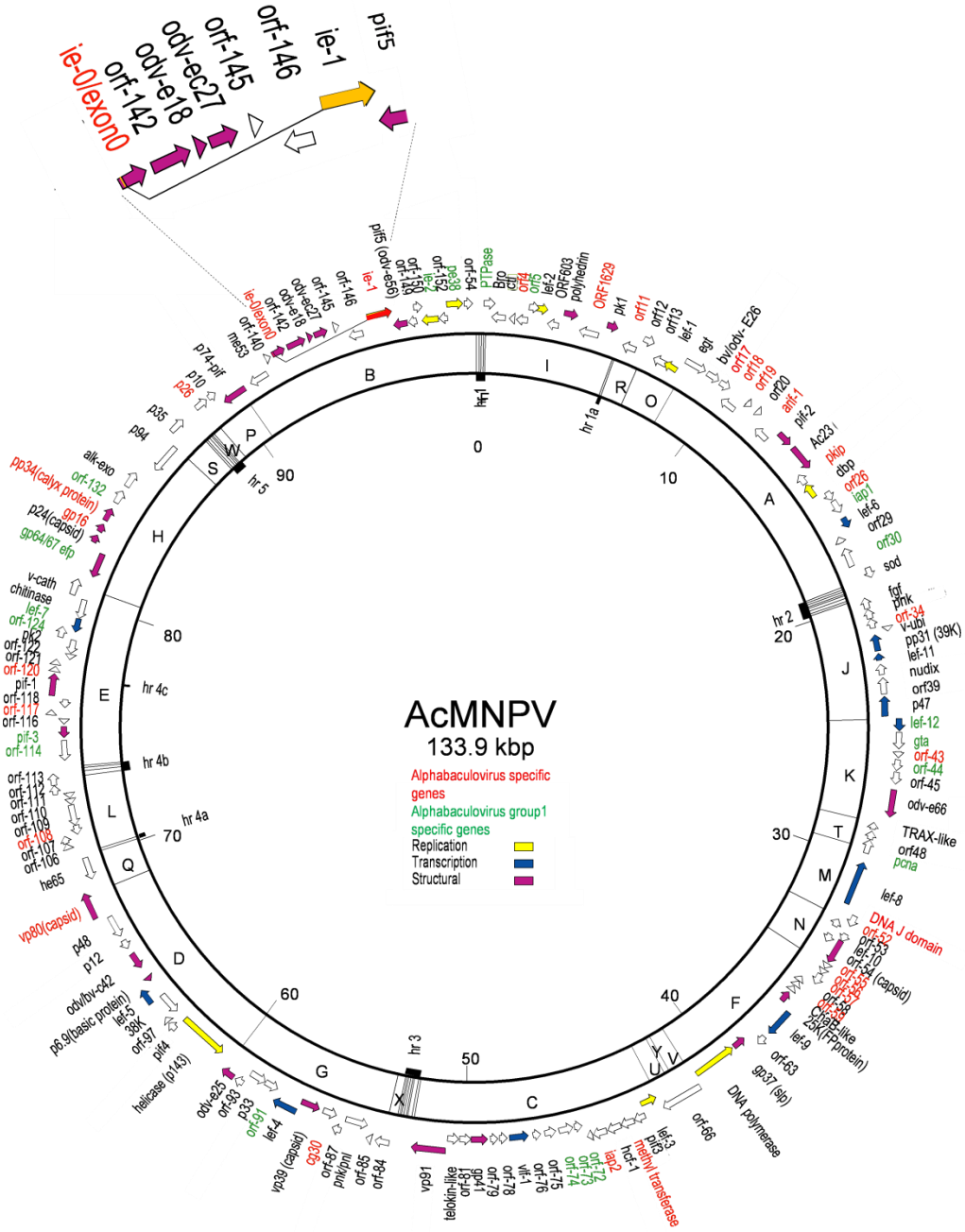

Figure 1. Organization of the AcMNPV genome. Baculoviruses have circular double stranded DNA genomes. The AcMNPV genome is 133.9 kbp and codes for 154 methionine initiated predicted open reading frames of 50 amino acids or larger. This study focuses on the *ie0-ie1* gene locus located within the inset noted in red. Reprinted from Encyclopedia of Virology 3rd edition, Theilmann D.A. and Blissard G.W., Baculoviruses: Molecular Biology of Nucleopolyhedroviruses, Pages No. 259 , Copyright (2008), with permission from Elsevier.

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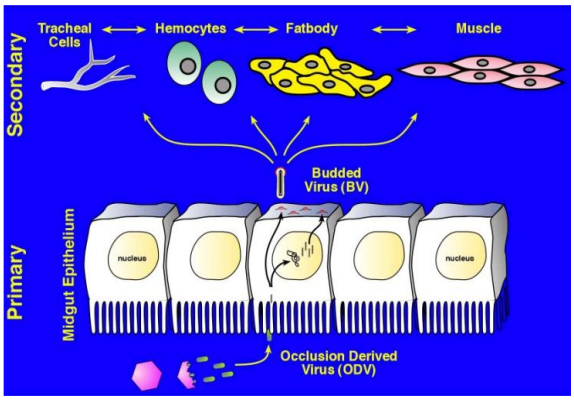

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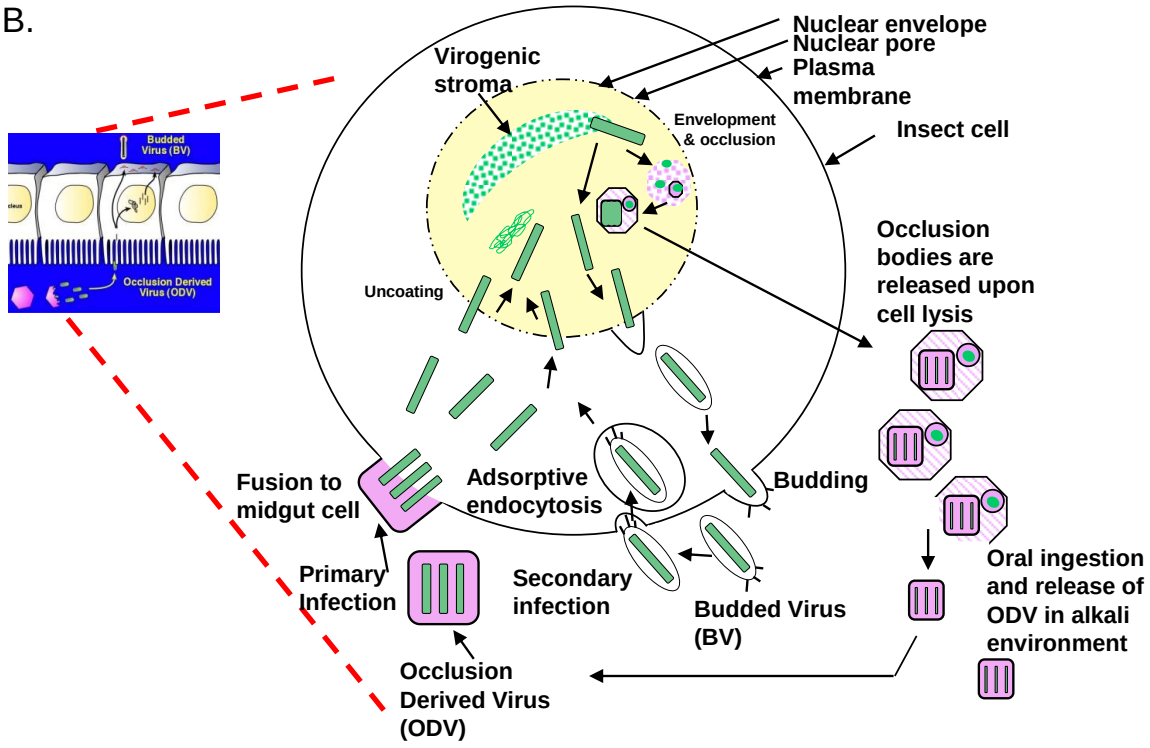


Figure 2: Alphabaculovirus life cycle. (A) ODV enter midgut epithelium cell and travel to the nucleus for viral replication or proceed to bud, resulting in secondary infection of tracheal cells, hemocytes, fatbody and muscle tissue. Adapted from Slack and Arif (2006). Reprinted from Encyclopedia of Virology 3rd edition, Theilmann D.A. and Blissard G.W., Baculoviruses: Molecular Biology of Nucleopolyhedroviruses, Pages No. 258 , Copyright (2008), with permission from Elsevier. (B) During primary infection ODV bind and fuse to the midgut cell, releasing nucleocapsids into the cytoplasm. The nucleocapsids are imported to the nucleus and uncoat their genome. In the nucleus, gene expression is initiated and viral DNA replication follows within the virogenic stroma. Nucleocapsid assembly occurs. Some nucleocapsids transit from the nucleus to the cytoplasm and bud through the cellular membrane producing BV, allowing secondary infection. Some nucleocapsids are enveloped and embedded within the polyhedrin matrix forming OB. OB are released upon cell lysis and remain in the environment until ingested by a host.

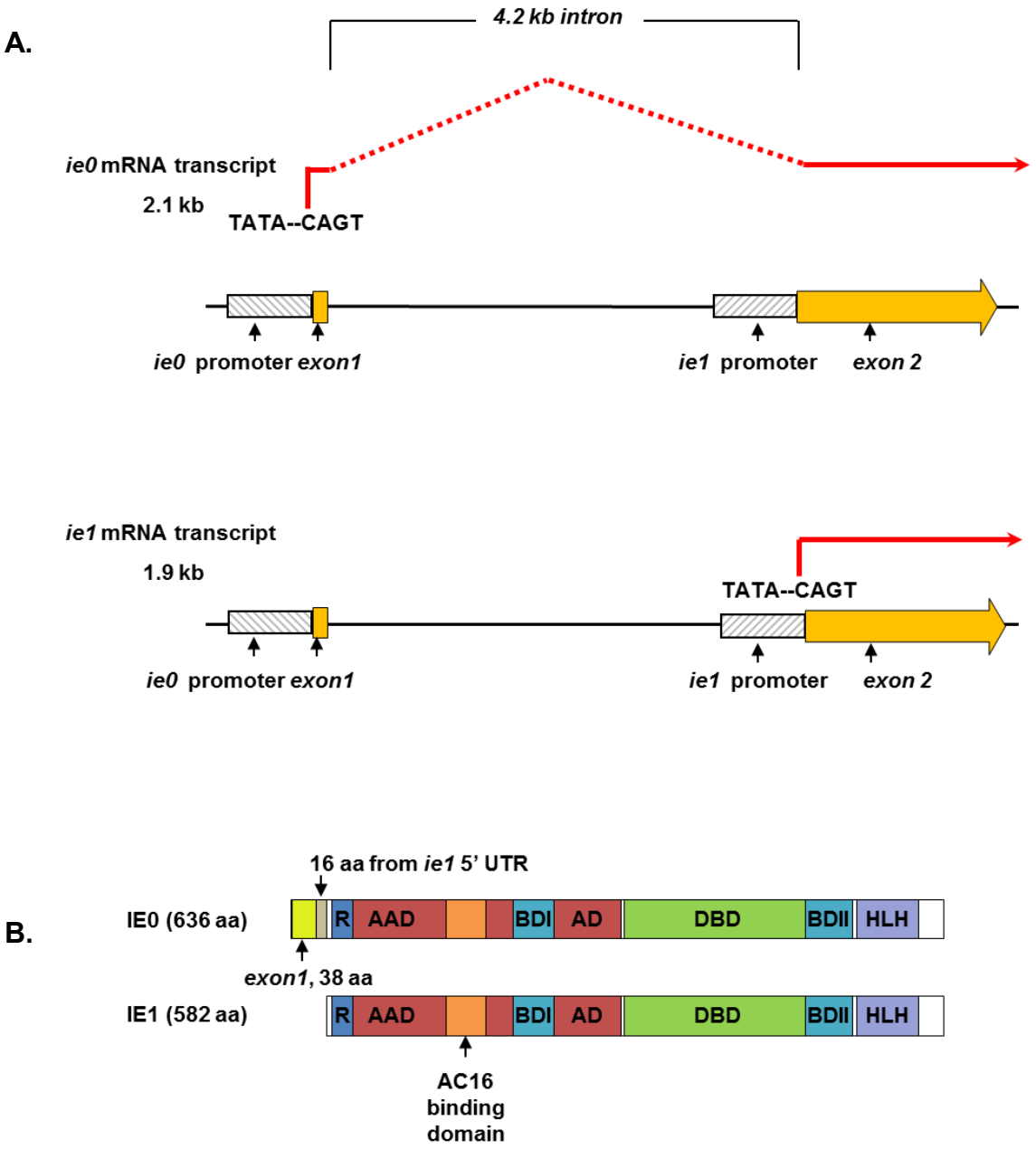


Figure 3: *ie0-ie1* gene complex and IE0/IE1 functional domains. (A) Schematic representation showing the mRNA transcripts of *ie0* and *ie1*. The *ie0* mRNA transcript is expressed under control of the *ie0* promoter and initiates 4.2 kb upstream at the "A" nucleotide of the CAGT *ie1* start site. The *ie0* mRNA transcript is composed of exon 1 and 2. The *ie1* mRNA transcript is expressed under control of the *ie1* promoter and is composed of only exon 2. (B) Functional domains of IE0 and IE1. Translation of the *ie1* mRNA transcript results in a protein containing the following domains: replication domain (R), acidic activation domain (AAD), AC16 binding domain, basic domain I (BDI), DNA binding domain (DBD), basic domain II (BDII) and helix- loop-helix motif (HLH). IE0 contains an additional 54 amino acids (aa), 38 aa coded by exon 1 and 16 aa coded by exon 2, coinciding with the 5' untranslated region (UTR) of the *ie1* mRNA transcript.

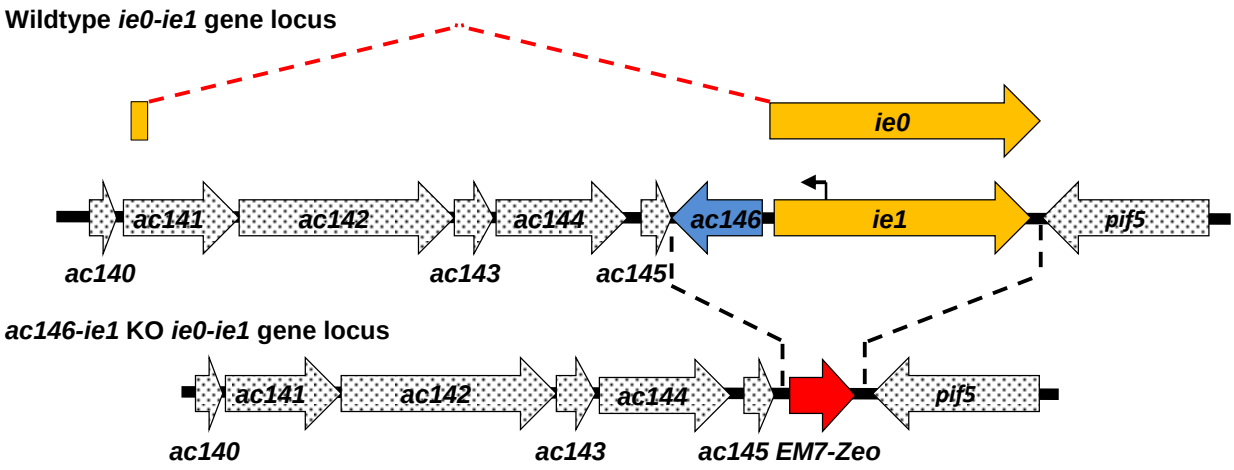


Figure 4: Construction of the *ac146-ie1* knock-out (KO) virus. The schematic diagram representing the AcMNPV wildtype *ie0-ie1* locus and the region replaced with a *zeocin* resistance cassette (*EM7-Zeo*) in the *ac146-ie1*KO virus (AcBac$^{ac146\text{-}ie1KO}$). The *ie0* ORF is composed *exon1* and *exon2* (orange arrow) separated by an intron (red dotted line), and the *ie1* ORF is only composed of *exon2* (orange box). The *ac146* ORF (blue arrow) is upstream of the *ie1* ORF and the late promoter for *ac146* (indicated by the arrow pointing to the left) resides within the *ie1* ORF. The ORFs of *ac140*, *ac141*, *ac142*, *ac143*, *ac144*, *ac145* and *odv-e56* are also located adjacent to the ie1 ORF and within the sequence spliced from the ie0 mRNA transcript. Replacement of the *ac146-ie1* locus by recombination with EM7 promoter-*zeocin* resistance gene (red arrow) deletes *ac146*, *ie1* and *ie0*.

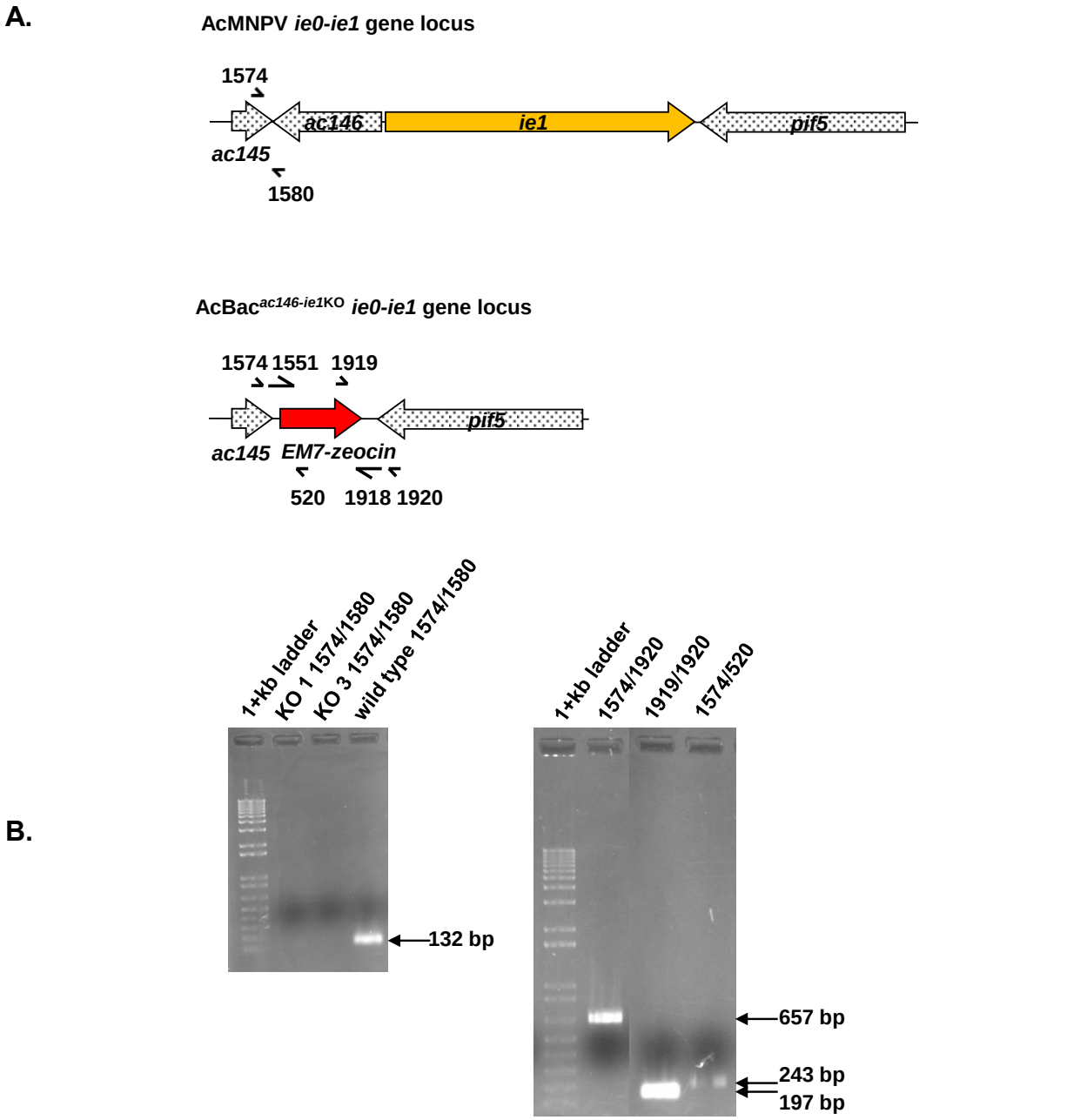


Figure 5: Confirmation of recombination events between wildtype bacmid and zeocin cassette at the *ac146-ie1* locus. (A) Diagram showing location of primers used to construct and confirm AcBac$^{ac146\text{-}ie1KO}$. Primers 1551 and 1918 were used to amplify the EM7-zeocin cassette and included homologous flanking regions adjacent to both 5' and 3' ends of the ac146-ie1 locus. The λ red recombinase method was used to insert the zeocin cassette into the AcMNPV E2 bacmid (bMON14272), in E. coli BW25113/pKD46 cells. Primers 1574 with 1580, 1574 with 1539, 1919 with 1920 and 1574 with 1920 were used to confirm the absence of the *ac146-ie1* and the presence of the zeocin cassette. (B) Agarose gel analysis of PCR products from two putative AcBac$^{ac146\text{-}ie1KO}$ clones (KO1 and KO3) and wildtype bacmid. Primers 1574 and 1580 were used to detect presence of *ac146-ie1* and wildtype bacmid DNA was used as a positive control. (C) Agarose gel analysis of AcBac$^{ac146\text{-}ie1KO}$ clone 1 (KO1) PCR products obtained using primer pairs to confirm the recombination cassette. To detect the 5' end of the zeocin cassette primers 1574 and 520 were used. To detect the 3' end of the zeocin cassette primers 1919 and 1920 were used. To detect the entire zeocin cassette, primers 1574 and 1920 were used.

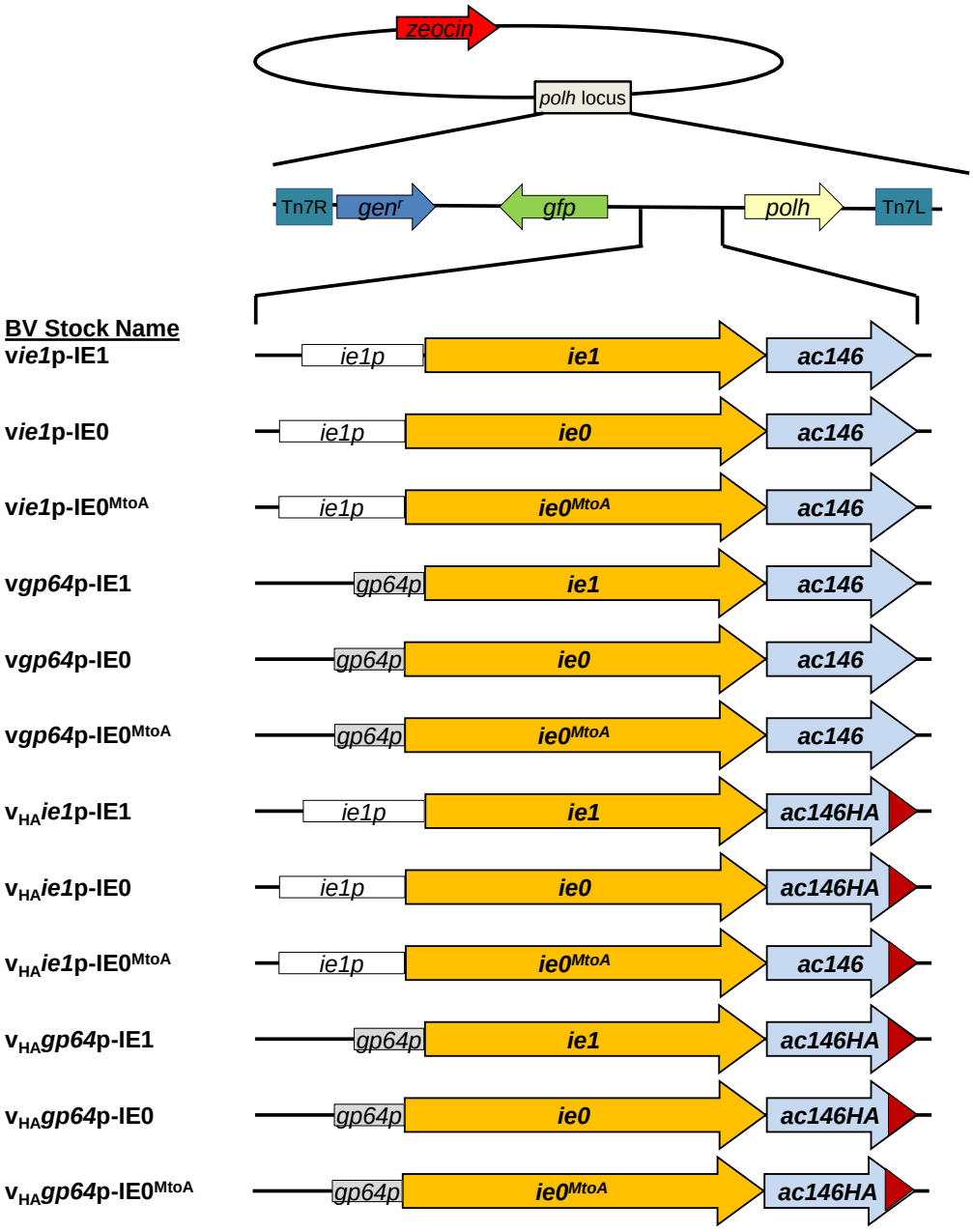


Figure 6: Construction of AcMNPV bacmid expressing *ie1*, *ie0*, or *ie0*^{MtoA} under control of the *ie1* or *gp64* promoters. The knockout bacmid AcBac$^{ac146\text{-}ie1KO}$ was made from AcMNPV E2 bacmid (bMON14272) and repaired with pFAcT-GFP transfer vectors. pFAcT-GFP vectors contain two transposition sites (Tn7R and Tn7L), the gentamicin resistance gene (*gen*^{R}), green fluorescence protein gene (*gfp*) and polyhedrin gene (*polh*) all located in the polyhedrin locus (*polh* locus). The knockout bacmid AcBac$^{ac146\text{-}ie1KO}$ was repaired by transposition to express *ie1, ie0* or *ie0*^{MtoA} under control of the *ie1* promoter (white box) or under control of the *gp64* promoter (grey box). Included in the repair vectors were *ac146* (blue arrow) or *ac146HA* (blue arrow with red tip). *Sf*9 cells were transfected with the repaired bacmids in duplicate and budded virus stocks were harvested. The budded virus harvested for each repaired bacmid were named as follows; v*ie1*p-IE1, v*ie1*p-IE0, v*ie1*p-IE0^{MtoA}, v*gp64*p-IE1, v*gp64*p-IE0, v*gp64*p-IE0^{MtoA}, v$_{HA}$*ie1*p-IE1, v$_{HA}$*ie1*p-IE0, v$_{HA}$*ie1*p-IE0^{MtoA}, v$_{HA}$*gp64*p-IE1, v$_{HA}$*gp64*p-IE0, and v$_{HA}$*gp64*p-IE0^{MtoA}.

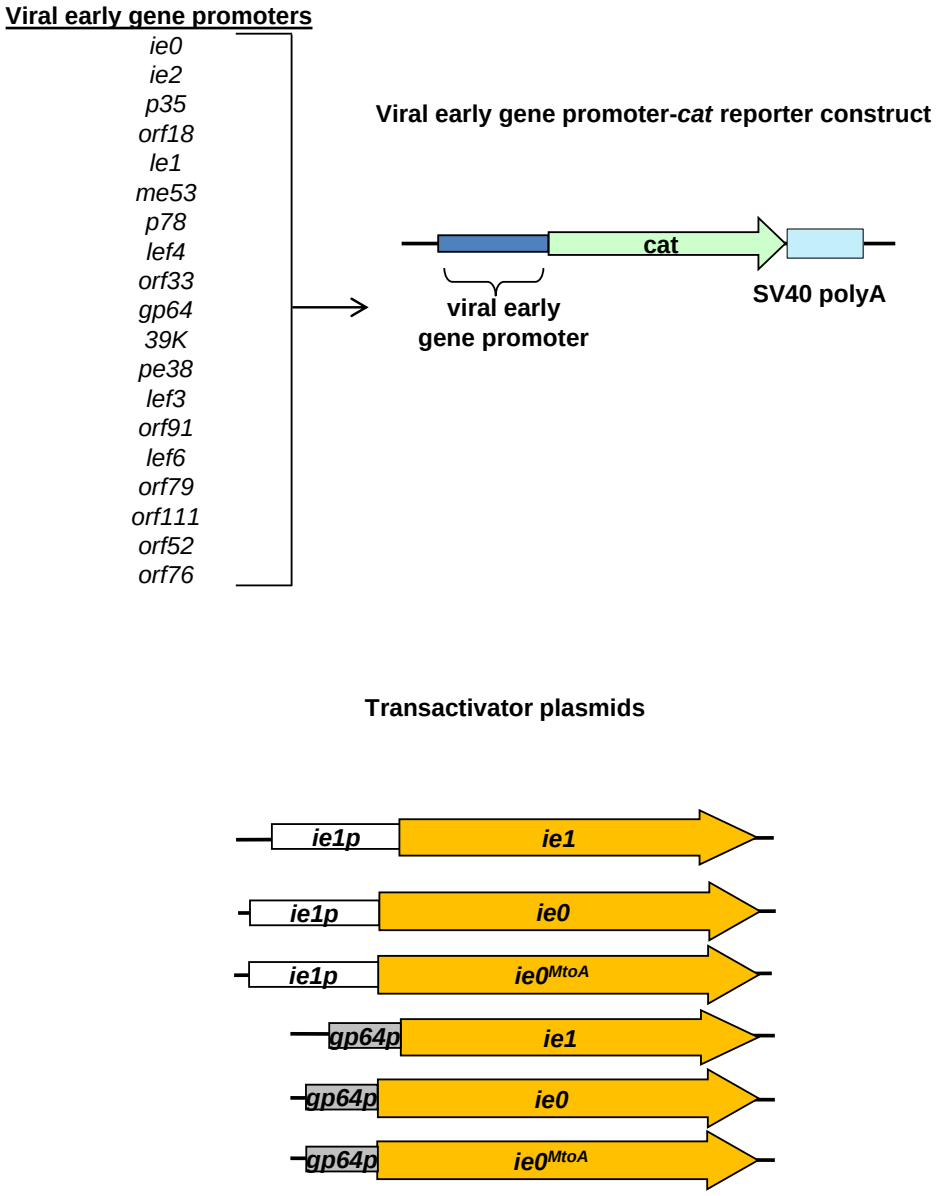


Figure 7: Schematic diagrams of plasmids used in transient transactivation co-transfection assays. The viral early gene promoter plasmids were constructed to have a viral early gene promoter controlling *cat* expression. The transactivator plasmids were constructed to have *ie0*, *ie0*^{MtoA} and *ie1* under control of the *ie1* or *gp64* promoter. Co-transfections of a viral early gene promoter plasmid and each transactivator plasmid allowed comparison of transactivation the viral early gene promoter by IE0 and IE1 under control of identical promoters. CAT assays were performed to measure the amount of CAT produced, as a way to measure promoter transactivation. Nineteen viral early gene promoter constructs were tested. Baseline level of CAT production was assessed by co-transfection of each viral early gene promoter and pBS+ plasmid.

Figure 8: Fluorescence microscopy analysis of transfected *Sf*9 cells (bacmids under control of *ie1* promoter). *Sf*9 cells were transfected in duplicate with wildtype bacmid, knockout bacmid AcBac$^{ac146\text{-}ie1\text{KO}}$, repair bacmids expressing *ie0*, *ie0*^{MtoA} or *ie1* under control of the *ie1* promoter. Cells were observed under fluorescence microscopy at 24 and 48 hpt. Increased GFP spread over time indicated viral replication and BV production in cells transfected with wildtype and all repaired bacmids. The knockout bacmid AcBac$^{ac146\text{-}ie1\text{KO}}$ did not show any increase in GFP expressing cells, suggesting no BV production.

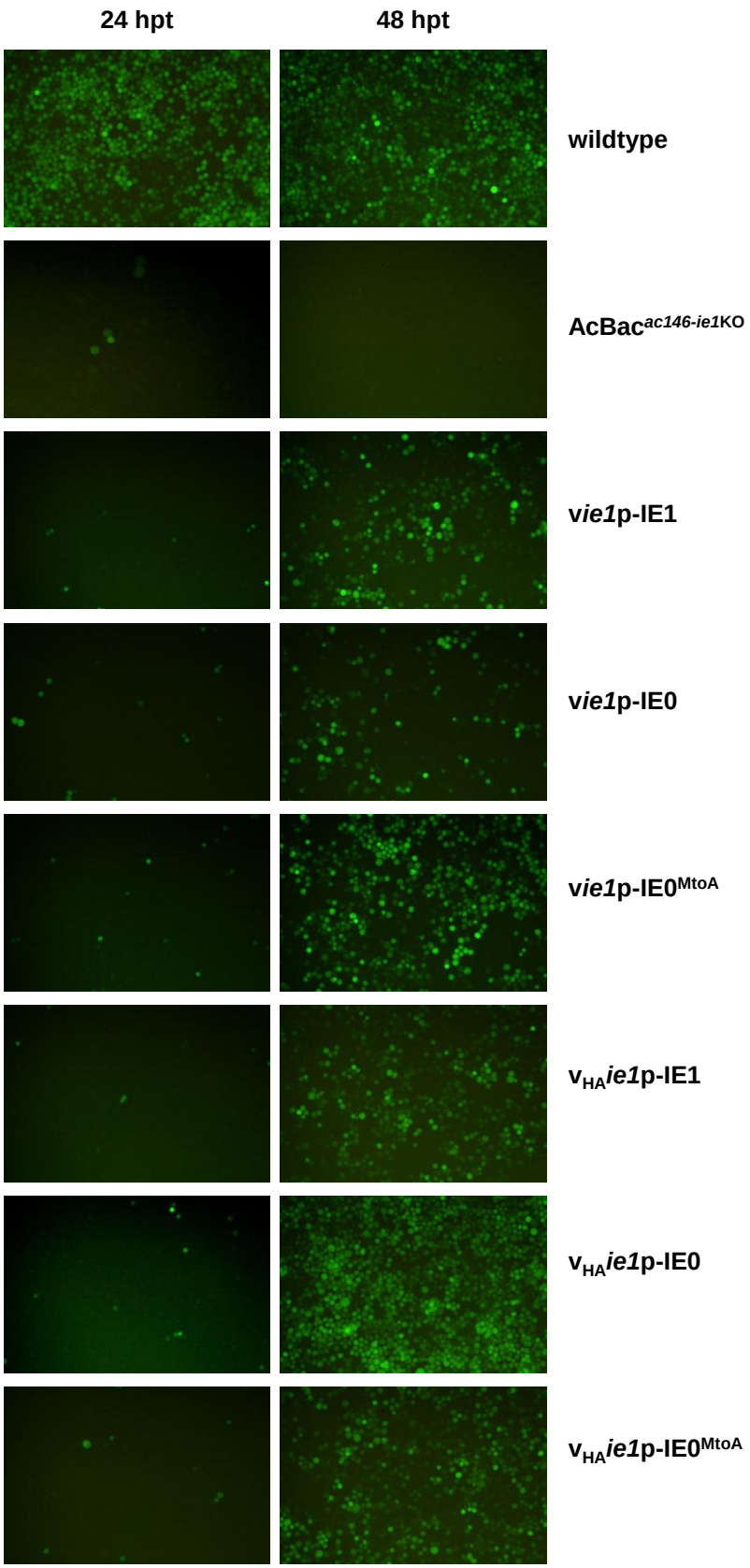
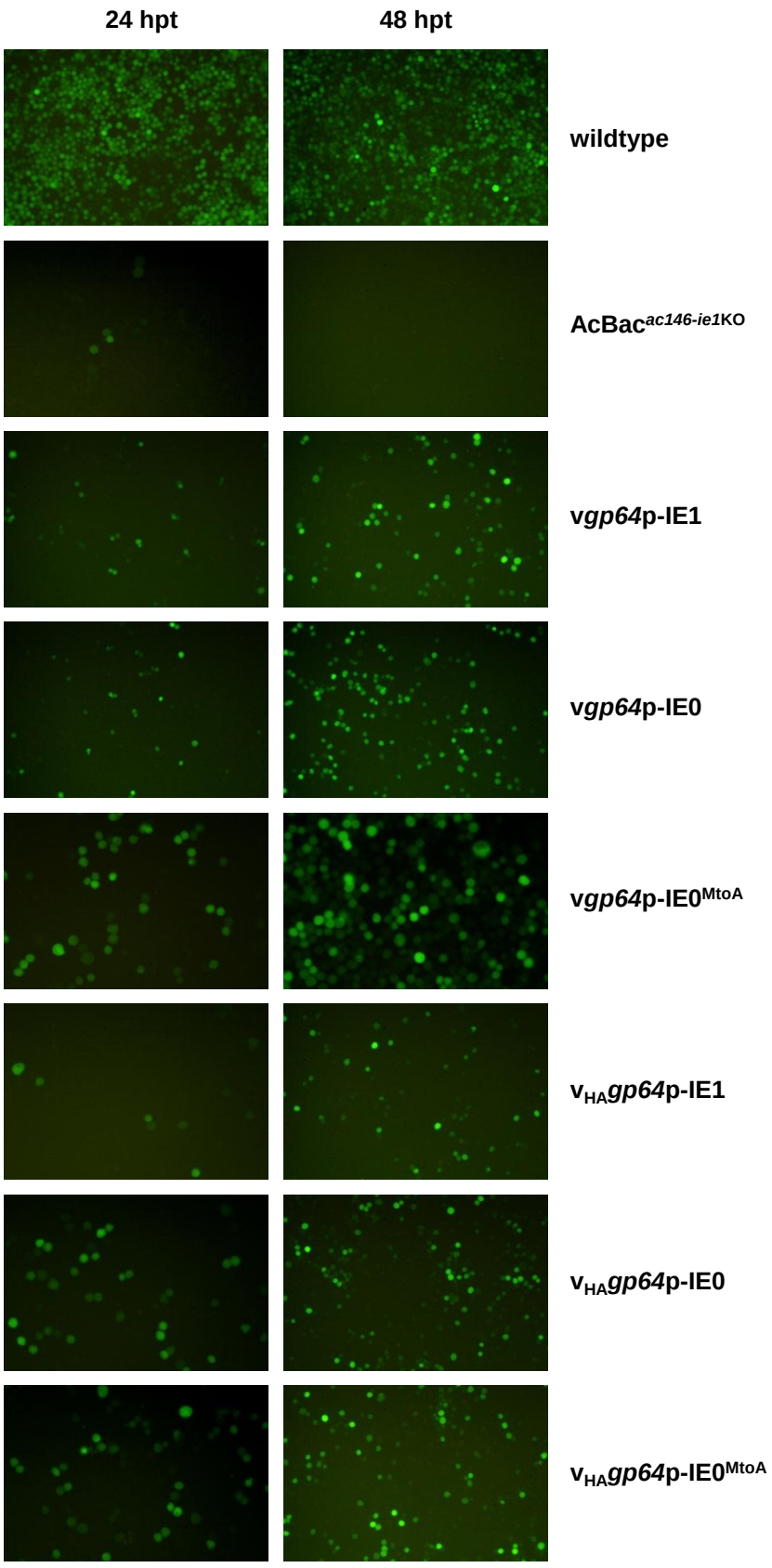

24 hpt
48 hpt
wildtype
AcBac$^{ac146\text{-}ie1KO}$
v*ie1*p-IE1
v*ie1*p-IE0
v*ie1*p-IE0^{MtoA}
v$_{HA}$*ie1*p-IE1
v$_{HA}$*ie1*p-IE0
v$_{HA}$*ie1*p-IE0^{MtoA}

Figure 9: Fluorescence microscopy analysis of transfected *Sf*9 cells (bacmids under control of *gp64* promoter). *Sf*9 cells were transfected in duplicate with wildtype bacmid, knockout bacmid AcBac$^{ac146\text{-}ie1KO}$, repair bacmids expressing *ie0*, *ie0*^{MtoA} or *ie1* under control of the *gp64* promoter. Cells were observed under fluorescence microscopy at 24 and 48 hpt. Increased GFP spread over time indicated viral replication and BV production in cells transfected with wildtype and all repaired bacmids. The knockout bacmid AcBac$^{ac146\text{-}ie1KO}$ did not show any increase in GFP expressing cells, suggesting no BV production.


24 hpt
48 hpt
wildtype
AcBac$^{ac146\text{-}ie1KO}$
v*gp64*p-IE1
v*gp64*p-IE0
v*gp64*p-IE0^{MtoA}
v$_{HA}$*gp64*p-IE1
v$_{HA}$*gp64*p-IE0
v$_{HA}$*gp64*p-IE0^{MtoA}

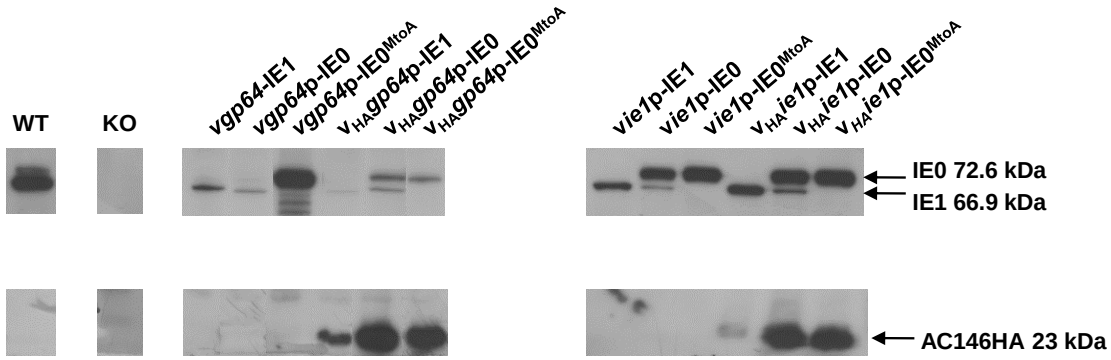


Figure 10: Western blot of IE0, IE1 and AC146HA expression in bacmid transfected *Sf*9 cells. At 48 hpt IE0, IE1 and AC146HA protein expression was analyzed for *Sf*9 cells transfected with wildtype (WT), AcBac$^{ac146\text{-}ie1KO}$ (KO), repair bacmids expressing *ie0*^{MtoA}, *ie0* or *ie1* under control of the *gp64* promoter or the *ie1* promoter. Total cell protein collected at 48 hpt was separated by 10% SDS-PAGE and transferred to membranes. IE0 and IE1 were detected with an anti-IE1 mouse monoclonal primary antibody. AC146HA was detected with an anti-HA mouse monoclonal primary antibody. Bound antibody was immunodetected using Western-Lightening® Plus ECL Enhanced Chemiluminescence System (Perkin-Elmer).

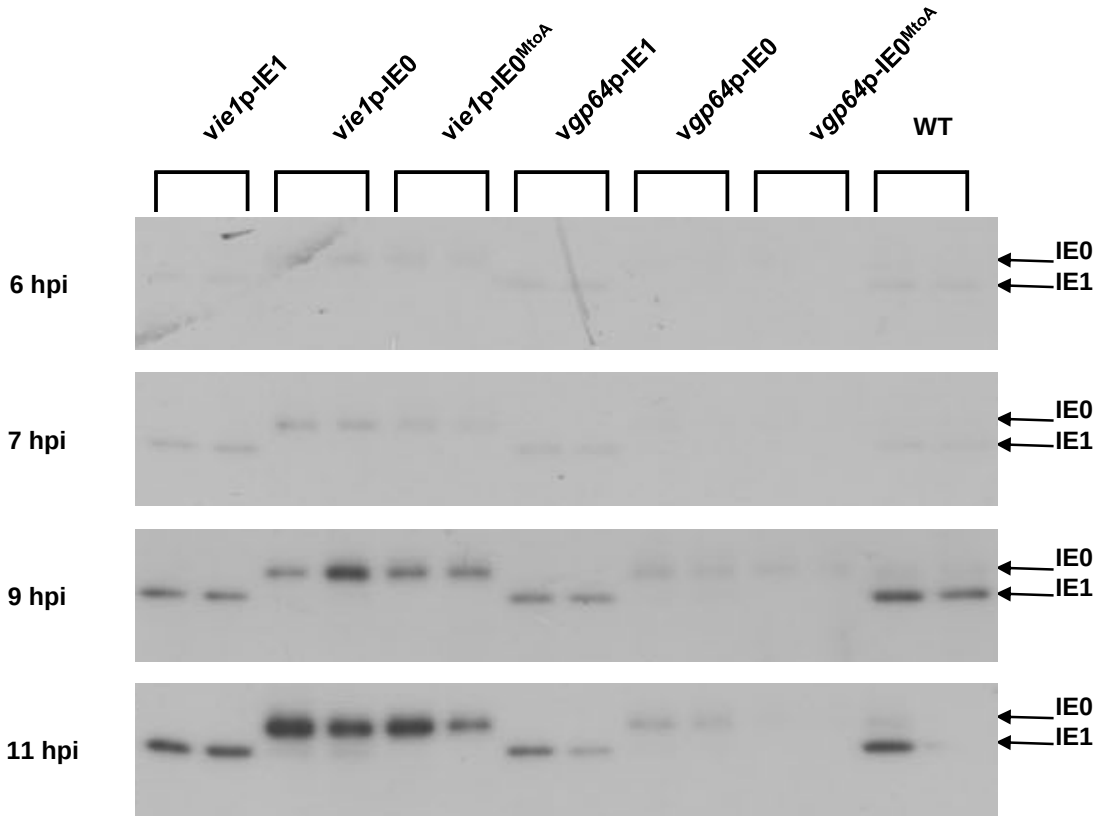


Figure 11: Western blot of IE0 and IE1 in virus infected *Sf*9 cells at early times post infection. *Sf*9 cells were infected at an MOI of 5 with budded virus in quadruplicate with recombinant viruses expressing *ie1*, *ie0* or $ie0^{MtoA}$ under control of the *ie1* promoter, recombinant virus expressing *ie1*, *ie0* or $ie0^{MtoA}$ under control of the *gp64* promoter and wildtype virus (WT). Total cell protein collected at 6, 7, 9, and 11 hpi was separated by 10% SDS-PAGE and transferred to membranes. IE0 and IE1 were detected with an anti-IE1 mouse monoclonal primary antibody. Bound antibody was immunodetected using Western-Lightening® Plus ECL Enhanced Chemiluminescence System (Perkin-Elmer).

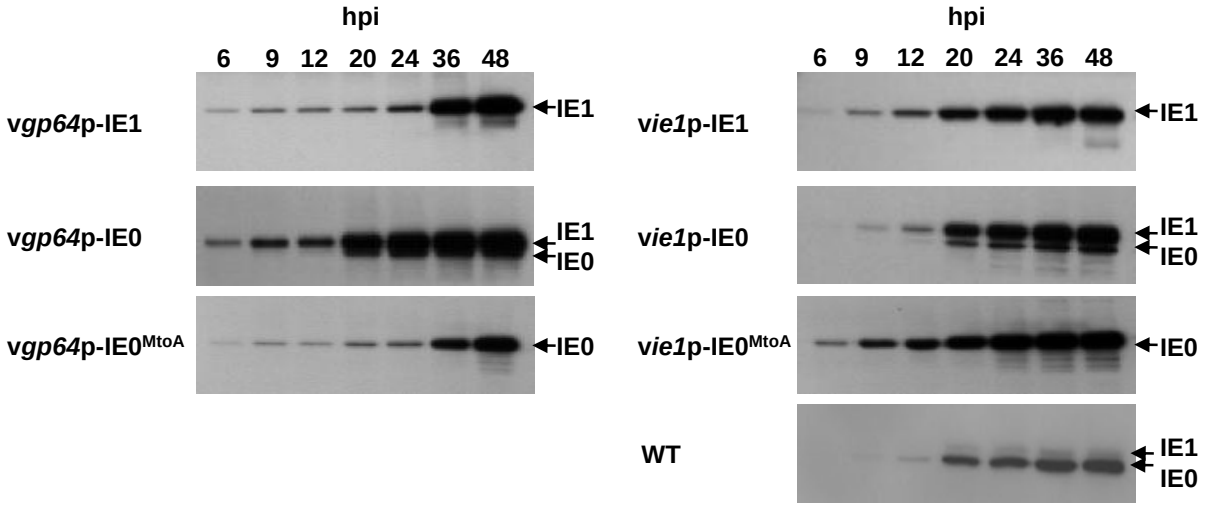


Figure 12: Western blot of IE0 and IE1 in virus infected *Sf*9 cells at representative times post infection. *Sf*9 cells were infected at an MOI of 5 with budded virus in quadruplicate with viruses expressing IE0, IE0^{MtoA} and IE1 under control of the *gp64* promoter (A) and *ie1* promoter (B). Total cell protein collected at 6, 9, 12, 20, 24, 36 and 48 hpi was separated by 10% SDS-PAGE and transferred to membranes. IE0 and IE1 were detected with an anti-IE1 mouse monoclonal primary antibody. Bound antibody was immunodetected using Western-Lightening® Plus ECL Enhanced Chemiluminescence System (Perkin-Elmer).

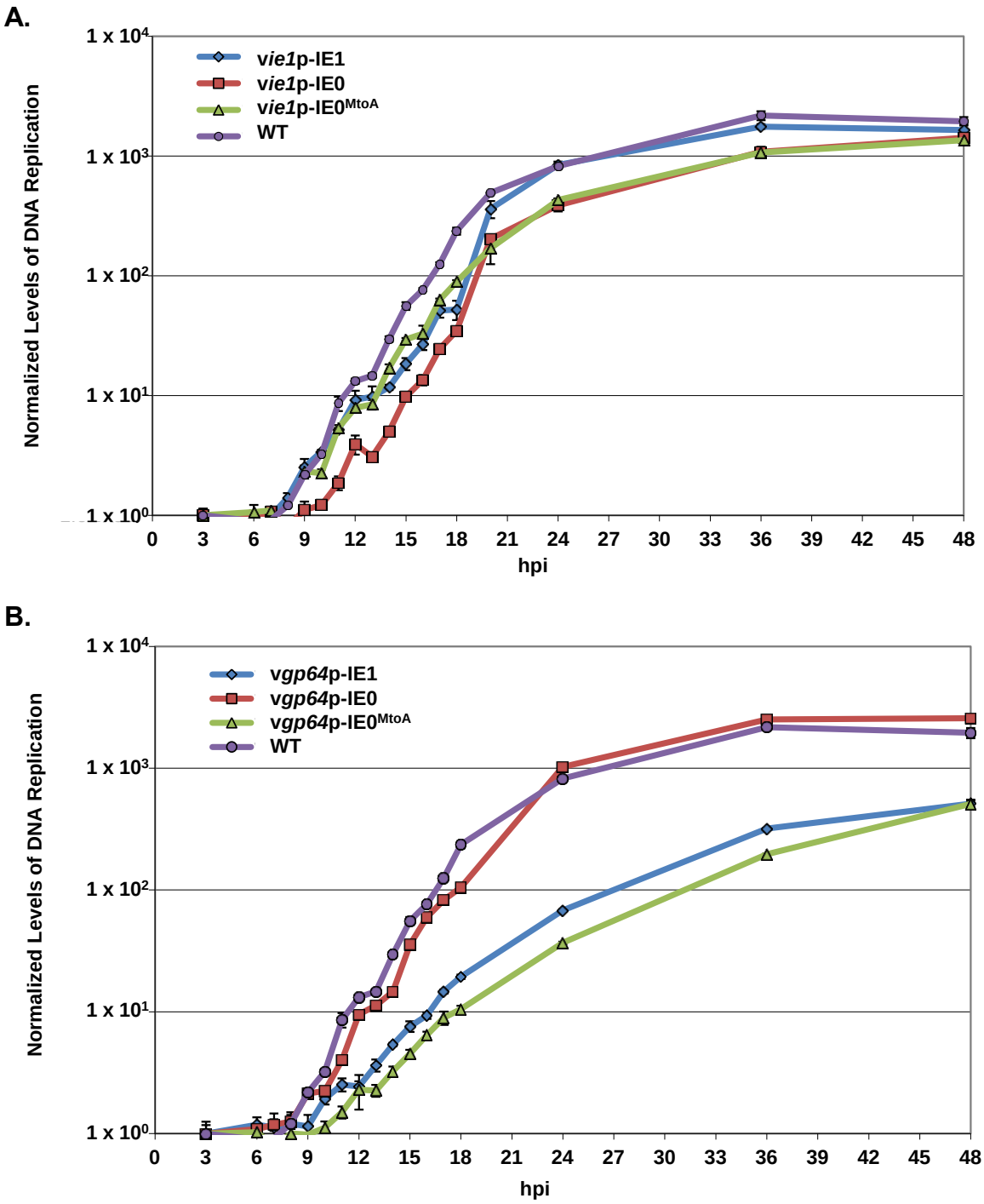


Figure 13: Time course analysis of viral DNA replication in infected *Sf*9 cells. *Sf*9 cells were infected at an MOI of 5 with budded virus in quadruplicate with (A) v*ie1*p-IE1, v*ie1*p-IE0, v*ie1*p-IE0^{MtoA}, and WT (B) v*gp64*p-IE1, v*gp64*p-IE0, v*gp64*p-IE0^{MtoA} and WT. Cell pellets were collected at 3 hpi, hourly time points between 6 and 18 hpi, as well as 24, 36 and 48 hpi. Viral DNA replication was assessed by qPCR and each data point represents the average of 2 to 4 assays. The level of DNA replication was normalized relative to 3 hpi for each virus and error bars represent standard error. When error bars are not visible they are smaller than the symbol used.

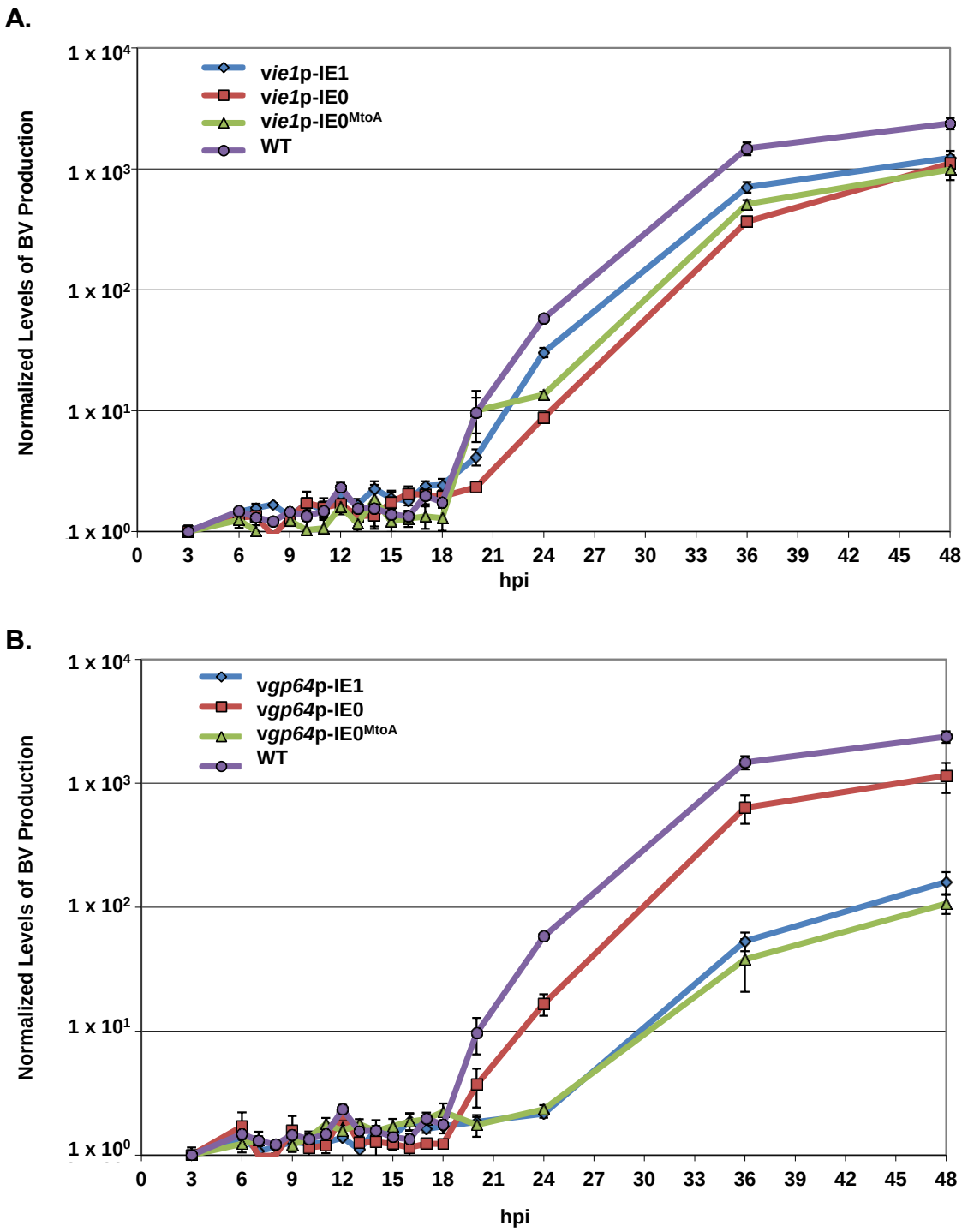


Figure 14: Viral BV production during time course assays. *Sf*9 cells were infected at an MOI of 5 with budded virus in quadruplicate with (A) v*ie1*p-IE1, v*ie1*p-IE0, v*ie1*p-IE0^{MtoA}, and WT (B) v*gp64*p-IE1, v*gp64*p-IE0, v*gp64*p-IE0^{MtoA} and WT. Supernatants were collected at 3 hpi, hourly time points between 6 and 18 hpi, as well as 24, 36 and 48 hpi. BV production was assessed by qPCR and each data point represents the average of 2 to 4 assays. The level of BV production was normalized relative to 3 hpi for each virus and error bars represent standard error. When error bars are not visible they are smaller than the symbol used.

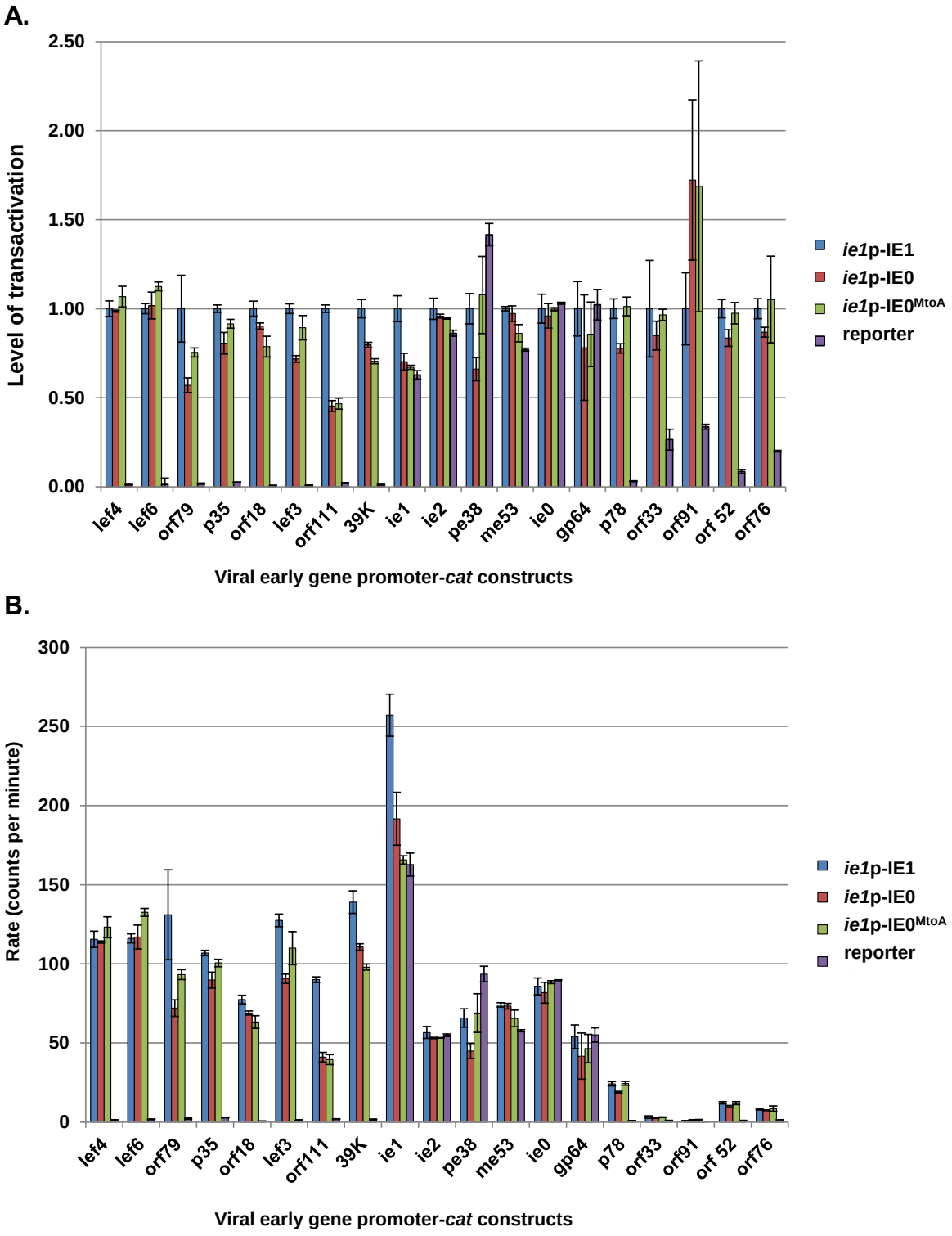


Figure 15: Transactivation analysis of AcMNPV early gene promoters by IE0 and IE1 under control of the *ie1* promoter. *Sf*9 cells were co-transfected with 0.5 µg of viral early gene promoter-*cat* constructs (indicated on the x axis), and 0.5 µg of transactivator plasmids with the *ie1* promoter driving expression *ie1* (purple), *ie0* (maroon), *ie0*^{MtoA} (yellow) or reporter alone without a transactivator (blue). (A) represents the levels of transactivation normalized relative to transactivation achieved with IE1. (B) represents the actual rates measured. Co-transfections were performed in duplicate and error bars represent standard error. When error bars are not visible they are too small to be distinguishable on the y axis.

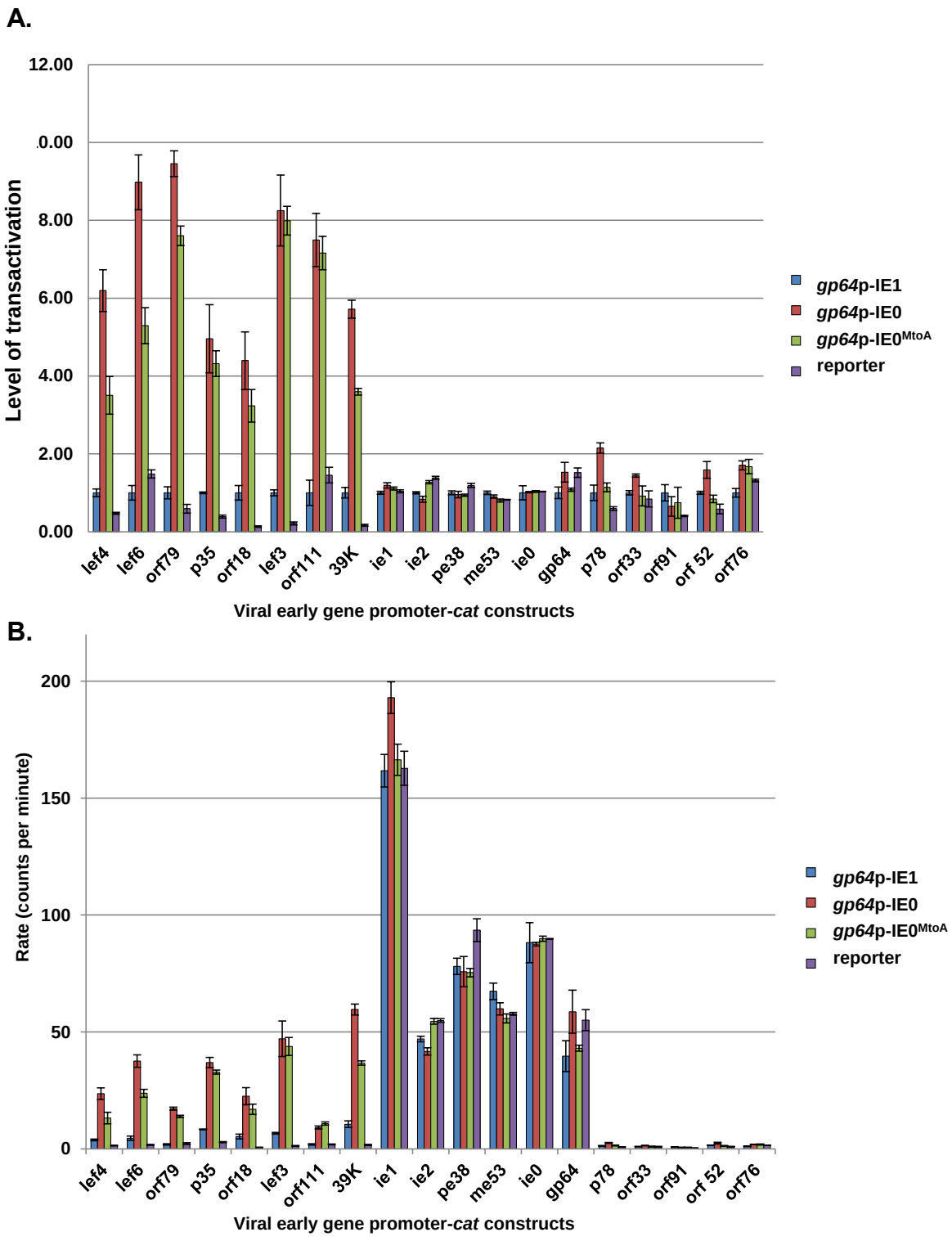


Figure 16: Transactivation analysis of AcMNPV early gene promoters by IE0 and IE1 under control of the *gp64* promoter. *Sf*9 cells were co-transfected with 0.5 µg of viral early gene promoter-*cat* constructs (indicated on the x axis), and 0.5 µg of transactivator plasmids with the *gp64* promoter driving expression IE1 (blue), IE0 (red), $IE0^{MtoA}$ (green) or reporter alone without a transactivator (purple. (A) represents the levels of transactivation normalized relative to transactivation achieved with IE1. (B) represents the actual rates measured. Co-transfections were performed in duplicate and error bars represent standard error. When error bars are not visible they are too small to be distinguishable on the y axis.

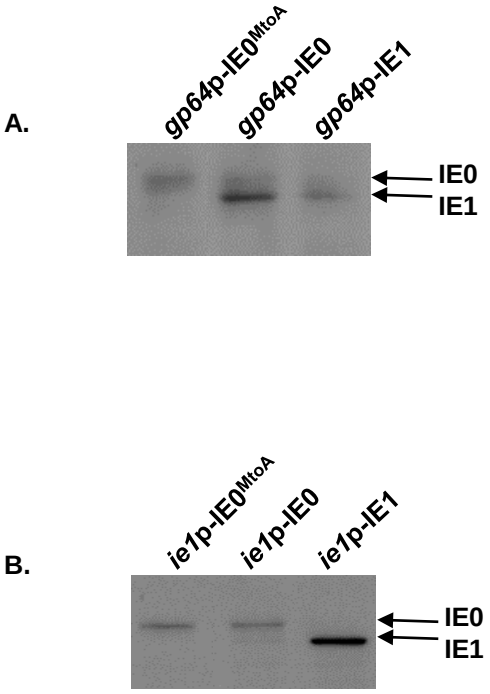
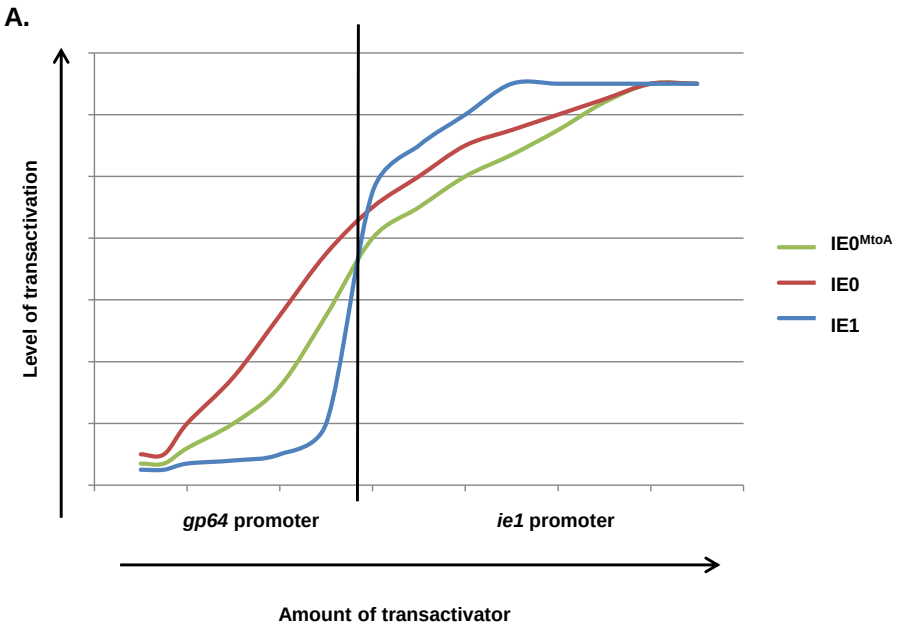


Figure 17: Western blot of IE0, IE0^{MtoA} and IE1 in transient co-transfected *Sf*9 cells. *Sf*9 cells were co-transfected with a viral early gene promoter-cat reporter plasmid and a transactivator plasmid and assayed for CAT activity at 48 hpt. Total protein from remaining samples were separated by 10% SDS-PAGE and transferred to membranes. IE0 and IE1 were detected with an anti-IE1 mouse monoclonal primary antibody. Bound antibody was immunodetected using Western-Lightening® Plus ECL Enhanced Chemiluminescence System (Perkin-Elmer). The Western blots represent samples with the following plasmids transfected (A) *p35* promoter-cat reporter plasmid and IE0, IE0^{MtoA}, or IE1 under control of the *gp64* promoter (B) *orf111* promoter-cat reporter plasmid and IE0, IE0^{MtoA} or IE1 under control of the *ie1* promoter.



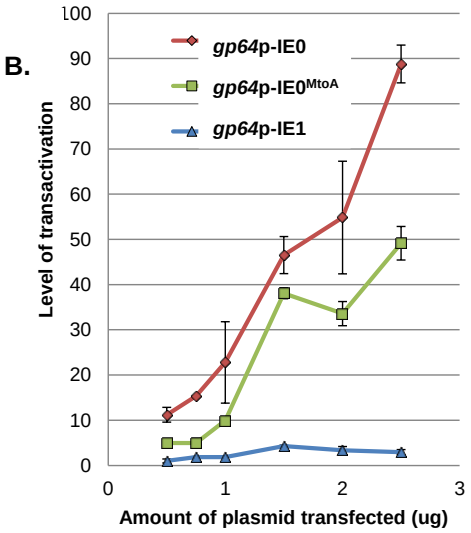


Figure 18: Preliminary analysis of amount of transactivator plasmid transfected and rate of transactivation. (A) Illustration of a model where transactivators may behave according to the observations seen when present at high and low levels. At low amounts of transactivator (*gp64* promoter), level of transactivation by IE0 and IE0^{MtoA} increases modestly as the amount of IE0 and IE0^{MtoA} increases. The level of transactivation achieved with IE0 is higher than IE0^{MtoA}. As amounts IE0 and IE0^{MtoA} increase (*ie1* promoter), the level of transactivation increases until the maximum level of transactivation is eventually reached. However, at low amounts of IE1 (*gp64* promoter), the level of transactivation increases slowly. Over a short range of amounts of IE1 transactivator, the level of transactivation by IE1 increases sharply, and at high levels of IE1 (*ie1* promoter), maximum transactivation is reached. In this example, maximum transactivation achieved when IE1 is present at levels less than IE0 and IE0^{MtoA}. (B) *Sf*9 cells were transfected with varying amounts of transactivator (x axis) and 0.5 µg of *39K* promoter-*cat* reporter plasmid. Cells were collected at 48 hpt and level of transaction was analyzed. Co-transfections were performed in duplicate and error bars represent standard error.

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Appendix A : Replicated randomized block ANOVA withTukeyHSD

The following script was used in R software to analyze transactivation data.

```
#Read in the Data
yfull=read.table('filen
ame.csv',header=F,sep
=',')
Block=as.factor(c(rep
('Day1',8),rep('Day2'
,8)))
Trt=c('ie1','ieo','ie0
MtoA','reporter')
Trt=as.factor(rep(Trt
,4))

y=c(3.6,3.5,12.7,13.5,25,16,53,53,19,16,47,48,2,1,5,4)
#For the First Response do
y=yfull[,1]
Exp1=data.frame(Tr
t=Trt,Block=Block,y
=y)
an1=aov(y~Trt+Bloc
k,data=Exp1)
summary(an1)
TukeyHSD(an1,'Trt')
#Second  Response is!
y=yfull[,2]
Exp1=data.frame(Tr
t=Trt,Block=Block,y
=y)
an1=aov(y~Trt+Bloc
k,data=Exp1)
summary(an1)
TukeyHSD(an1,'Trt')
y=yfull[,3]
Exp1=data.frame(Tr
t=Trt,Block=Block,y
=y)
```

```
an1=aov(y~Trt+Bloc
k,data=Exp1)
summary(an1)
TukeyHSD(an1,'Trt')
y=yfull[,4]
Exp1=data.frame(Tr
t=Trt,Block=Block,y
=y)
an1=aov(y~Trt+Bloc
k,data=Exp1)
summary(an1)
TukeyHSD(an1,'Trt')
y=yfull[,5]
Exp1=data.frame(Tr
t=Trt,Block=Block,y
=y)
an1=aov(y~Trt+Bloc
k,data=Exp1)
summary(an1)
TukeyHSD(an1,'Trt')
y=yfull[,6]
Exp1=data.frame(Tr
t=Trt,Block=Block,y
=y)
an1=aov(y~Trt+Bloc
k,data=Exp1)
summary(an1)
TukeyHSD(an1,'Trt')
y=yfull[,7]
Exp1=data.frame(Tr
t=Trt,Block=Block,y
=y)
an1=aov(y~Trt+Bloc
k ,data=Exp1)
summary(an1)
TukeyHSD(an1,'Trt')
```

```
y=yfull[,8]
Exp1=data.frame(Tr
t=Trt,Block=Block,y
=y)
an1=aov(y~Trt+Bloc
k,data=Exp1)
summary(an1)
TukeyHSD(an1,'Trt')
y=yfull[,9]
Exp1=data.frame(Tr
t=Trt,Block=Block,y
=y)
an1=aov(y~Trt+Bloc
k,data=Exp1)
summary(an1)
TukeyHSD(an1,'Trt')
y=yfull[,10]
Exp1=data.frame(Tr
t=Trt,Block=Block,y
=y)
an1=aov(y~Trt+Bloc
k,data=Exp1)
summary(an1)
TukeyHSD(an1,'Trt')
y=yfull[,11]
Exp1=data.frame(Tr
t=Trt,Block=Block,y
=y)
an1=aov(y~Trt+Bloc
k,data=Exp1)
summary(an1)
TukeyHSD(an1,'Trt')
y=yfull[,12]
Exp1=data.frame(Tr
t=Trt,Block=Block,y
=y)
an1=aov(y~Trt+Bloc
```

```
k,data=Exp1)
summary(an1)
TukeyHSD(an1,'Trt')
y=yfull[,13]
Exp1=data.frame(Tr
t=Trt,Block=Block,y
=y)
an1=aov(y~Trt+
Block,data=Exp
1)
summary(an1)
TukeyHSD(an1,'
Trt')
y=yfull[,14]
Exp1=data.frame(Tr
t=Trt,Block=Block,y
=y)
an1=aov(y~Trt+Bloc
k,data=Exp1)
summary(an1)
TukeyHSD(an1,'Trt')
y=yfull[,15]
Exp1=data.frame(Tr
t=Trt,Block=Block,y
=y)
an1=aov(y~Trt+Bloc
k,data=Exp1)
summary(an1)
TukeyHSD(an1,'Trt')
y=yfull[,16]
Exp1=data.frame(Tr
t=Trt,Block=Block,y
=y)
an1=aov(y~Trt+Bloc
k,data=Exp1)
summary(an1)
TukeyHSD(an1,'Trt')
```

```
y=yfull[,17]
Exp1=data.frame(Tr
t=Trt,Block=Block,y
=y)
an1=aov(y~Trt+Bloc
k,data=Exp1)
summary(an1)
TukeyHSD(an1,'Trt')
y=yfull[,18]
Exp1=data.frame(Tr
t=Trt,Block=Block,y
=y)
an1=aov(y~Trt+Bloc
k,data=Exp1)
summary(an1)
TukeyHSD(an1,'Trt')
y=yfull[,19]
Exp1=data.frame(Tr
t=Trt,Block=Block,y
=y)
an1=aov(y~Trt+Bloc
k,data=Exp1)
summary(an1)
TukeyHSD(an1,'Trt')
```