Virology171 468-470 (2014) 160–171

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Defining the roles of the baculovirus regulatory proteins IE0 and IE1 in genome replication and early gene transactivation

Nadia Sokal [c], Yingchao Nie [a,1], Leslie G. Willis [a], Junya Yamagishi [b,2], Gary W. Blissard [b], Mark R. Rheault [c], David A. Theilmann [a,c,*]

[a] Pacific Agri-Food Research Centre, AAFC, Summerland, British Columbia, Canada V0H 1Z0
[b] Boyce Thompson Institute at Cornell University, Ithaca, New York, 14853-1801 USA
[c] Department of Biology, University of British Columbia, Kelowna, British Columbia, Canada V1V 1V7

ARTICLE INFO

Article history:
Received 2 July 2014
Returned to author for revisions
15 July 2014
Accepted 17 July 2014
Available online 30 August 2014

Keywords:
Baculovirus
AcMNPV
IE0
IE1
DNA replication
Transactivation
Gene expression
Transcription factor

ABSTRACT

IE0 and IE1 of the baculovirus *Autographa californica* multiple nucleopolyhedrovirus are essential transregulatory proteins required for both viral DNA replication and transcriptional transactivation. IE0 is identical to IE1 except for 54 amino acids at the N-terminus but the functional differences between these two proteins remain unclear. The purpose of this study was to determine the separate roles of these critical proteins in the virus life cycle. Unlike prior studies, IE0 and IE1 were analyzed using viruses that expressed *ie0* and *ie1* from an identical promoter so that the timing and levels of expression were comparable. IE0 and IE1 were found to equally support viral DNA replication and budded virus (BV) production. However, specific viral promoters were selectively transactivated by IE0 relative to IE1 but only when expressed at low levels. These results indicate that IE0 preferentially transactivates specific viral genes at very early times post-infection enabling accelerated replication and BV production.

Introduction

The immediate early proteins IE0 and IE1 are the key transregulatory proteins in the alphabaculovirus replication cycle. In the type species of the alphabaculoviruses, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), both IE0 and IE1 have been shown to transcriptionally regulate 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; Nissen and Friesen, 1989; Olson et al., 2001; Olson et al., 2002; Passarelli and Miller, 1993) as well as function as replication factors (Kool et al., 1994a; Luria et al., 2012; Pathakamuri and Theilmann, 2002; Stewart et al., 2005; Taggart et al., 2012). IE0 and IE1 are translated from distinct *ie0* and *ie1* mRNA transcripts which arise from spliced or unspliced mRNAs generated from two discrete promoters of the *ie0–ie1* gene complex. Additional spliced viral RNAs have recently been identified (Chen et al., 2013) but the *ie0–ie1* gene complex is still the only known spliced gene within the baculovirus genome that is processed into multiple protein products (Chisholm and Henner, 1988; Theilmann et al., 2001). The splicing event results in IE0 being identical to IE1 except for an additional 54 amino acids of no known function at its N-terminus (Chisholm and Henner, 1988). The presence of either IE0 or IE1 is essential for viral replication but both proteins are required for a wildtype infection (Stewart et al., 2005). Both IE0 and IE1 are present throughout the AcMNPV replication cycle, although their levels of expression are not equal. IE0 expression and abundance peaks during the first few hours post-infection prior to the initiation of DNA replication and declines thereafter (Huijskens et al., 2004). IE1 becomes more abundant than IE0 by the time replication begins and continues to increase throughout infection (Chen et al., 2013; Choi and Guarino, 1995b; Huijskens et al., 2004; Theilmann and Stewart, 1991). One of the most surprising characteristics of the spliced *ie0* transcript is that it is translated as both IE0 and IE1 due to internal translation initiation at the *ie1* start codon resulting in both proteins always being present (Theilmann et al., 2001). The reason that alphabaculoviruses

* Corresponding author at: Pacific Agri-Food Research Centre, AAFC, Box 5000, Summerland, British Columbia, Canada V0H 1Z0.
E-mail address: david.theilmann@agr.gc.ca (D.A. Theilmann).
[1] Current address: Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA, USA.
[2] Current address: Tohoku Medical Megabank Organization, Tohoku University, Sendai, Miyagi 980-8579, Japan.

http://dx.doi.org/10.1016/j.virol.2014.07.044
0042-6822/

produce IE0 in addition to IE1 is not clear, although the conservation of IE0 in the alphabaculoviruses suggests that this form of the protein serves an important or unique role. Both proteins appear to have similar transregulatory roles but no unique function has been identified for either protein. However, because both proteins must be present to achieve wildtype levels and progression of infection, it is important to understand the functional roles of both proteins, to fully understand baculovirus pathology.

IE0 or IE1 can transactivate genes in an enhancer-dependent or independent manner (Nissen and Friesen, 1989; Rodems and Friesen, 1993; Theilmann and Stewart, 1991). However, 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). In the absence of enhancer elements, IE0 and IE1 transactivate many early genes in transient assays but no specific IE0 or IE1 responsive element within the early gene promoters has been identified. 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. IE0 and IE1 form IE0-IE0 and IE1-E1 homodimers and IE0-IE1 heterodimers and all three dimer forms bind to enhancer elements (Kremer and Knebel-Morsdorf, 1998; Olson et al., 2001). In all prior comparisons of IE0 and IE1 transactivation, the two proteins were expressed under the control of their native promoters, resulting in a different temporal pattern of expression and differing levels of expression. It is unknown whether IE0 and IE1 transactivate promoters with the same efficiency.

IE0 or IE1 is also required for AcMNPV DNA replication, in conjunction with the replication factors LEF1, LEF2, LEF3, LEF11, viral DNA polymerase (DNApol) and helicase. Viral DNA replication is also augmented by the non-essential factors P35, LEF7, IE2 and PE38 (Kool et al., 1994a, 1995, 1994b; Lin and Blissard, 2002; Luria et al., 2012). In infected cells IE1 binds to *hr* elements and co-localizes in nuclear structures, thought to be viral replication factories (Kawasaki et al., 2004; Nagamine et al., 2006; Okano et al., 1999). Within these structures 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, 1991; Choi and Guarino, 1995a; Lu and Carstens, 1993; Mikhailov, 2003; Rodems et al., 1997).

One of the functional domains within IE0 and IE1 is an N-terminal transcriptional acidic activation domain (AAD) which also contains a domain essential for viral DNA replication (Pathakamuri and Theilmann, 2002; Taggart et al., 2012). The replication domain contains a motif that resembles a cyclin-dependent phosphorylation site (TPXR/H) and amino acid substitution in this region caused loss of DNA replication activity (Taggart et al., 2012). Interestingly, the ability to support viral DNA replication is not maintained when the *Orgyia pseudotsugata* MNPV IE1 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). If the replication domain is inactivated, IE1 can 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.

Past studies have shown that IE0 and IE1 both support viral DNA replication, but appear to play different roles. Recombinant viruses expressing only IE0 show a delay in onset of DNA replication compared to wildtype, but viral DNA accumulates to higher levels at later times post-infection. Whereas viruses expressing only IE1 initiate DNA replication and attain levels similar to wildtype virus (Stewart et al., 2005). In these experiments however, IE0 and IE1 were expressed under the control of their native promoters resulting in different temporal kinetics and expression levels of each protein. The impact observed on viral DNA replication could therefore be simply due to quantitative expression differences potentially masking any functional differences between the two proteins.

In this study, to determine the functional differences between IE0 and IE1 they were analyzed and compared by expressing both genes under control of the same promoter. This approach permitted similar temporal expression and similar levels of protein at very early times of post-infection. Results showed that IE0 and IE1 equally supported BV production and DNA replication in virus infected cells. However, significant differences were observed in transient transactivation studies which showed for the first time that IE0 preferentially transactivates a subset of viral early gene promoters.

Results

Construction of ie0 and ie1 knockout viruses and repair viruses

To investigate the function of IE0 and IE1, a *ie0–ie1* knockout virus (AcBac$^{ac146\text{-}ie1KO}$) was made to serve as a backbone for the construction of viruses expressing *ie0*, *ie0*^{MtoA} or *ie1* under control of the *gp64* promoter to achieve similar levels of IE0 and IE1 expression. The knockout deleted the entire *ie1* ORF, which also results in the deletion of *ie0*. The deletion of *ie1* ORF also deletes the promoter of the essential gene *ac146* (Dickison et al., 2012) which is contained within the *ie1* ORF (Fig. 1A). To account for this overlap, the complete ORFs of both *ac146* and *ie1* were deleted by replacement of *ac146-ie1* with an EM7-promoter-*zeocin* cassette by homologous recombination and the *ac146* ORF was reinserted into all repair viruses (Fig. 1A).

To compare the function of IE0 and IE1, the *ie0–ie1* knockout bacmid, AcBac$^{ac146\text{-}ie1KO}$, was repaired with a series of constructs containing either *ie0*, *ie0*^{MtoA} or *ie1* under control of the *gp64* promoter generating the viruses, v*gp64p*-IE1 which only produces IE1, v*gp64p*-IE0 which produces both IE0 and IE1, and v*gp64p*-IE0^{MtoA} which only produces IE0 because the IE1 initiation codon Met was changed to Ala (Fig. 1A). The *gp64* promoter was chosen because it is an immediate-early promoter that would permit the same temporal expression of IE0 and IE1 as it is constitutively active in insect cells (Blissard and Rohrmann, 1991; Chisholm and Henner, 1988; Guarino and Summers, 1988) and the AcMNPV promoter has been shown to be unaffected by IE0 or IE1 except for basal level transactivation (Nie, 2010). The repair viruses also inserted the *polyhedrin* (*polh*) gene and the *green fluorescence protein* (*gfp*) marker protein gene (Fig. 1A). The viral bacmids were transfected into *Spodoptera frugiperda clone 9 (Sf9)* cells to confirm virus viability and to confirm correct expression of IE0 and IE1 by Western blot. BV stocks were generated from bacmid transfected cells, titred and used for further experiments.

Expression of IE0 and IE1 under control of the gp64 promoter

To compare the temporal expression of IE0 and IE1 a time course of infection was performed and the expression of IE0 and IE1 was analyzed from each of the viruses plus wildtype (Fig. 1B). Expression of both IE1 and IE0 from v*gp64p*-IE1 and v*gp64p*-IE0^{MtoA} followed the same temporal pattern that is steadily increasing in expression levels up to 48 hpi with the largest increase between 24 and 36 hpi. Expression of IE0 and IE1 from v*gp64p*-IE0 differed from the other two viruses in that it had a more rapid increase in levels starting between 12 and 24 hpi. During the early period prior to, and concomitant with initiation of

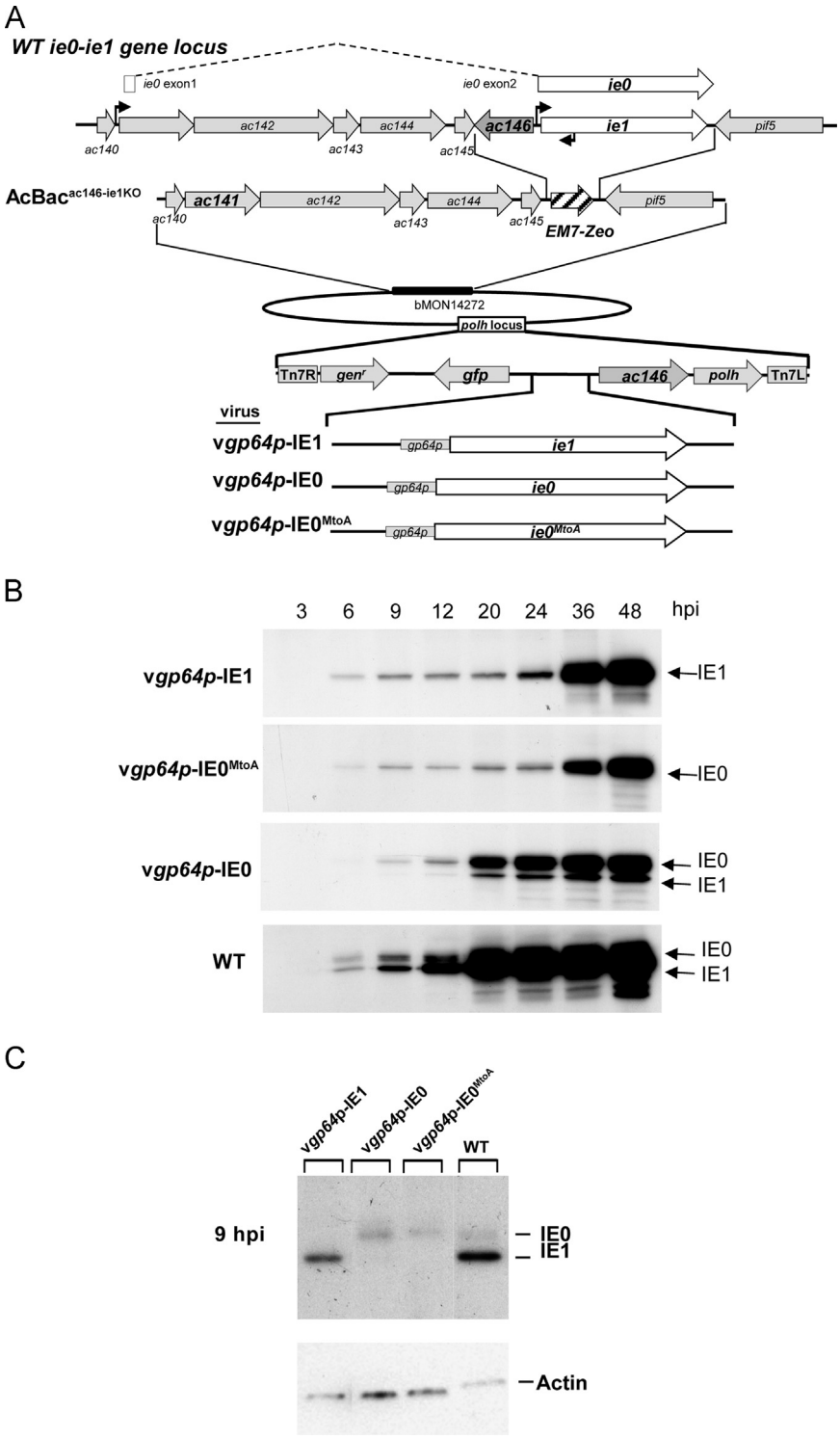

Fig. 1. Construction of viruses expressing *ie1*, *ie0*, or *ie0*^{MtoA} under control of the *gp64* promoters. (A) The AcMNPV wildtype *ie0–ie1* locus shows the *ie0* ORF which is composed *exon1* and *exon2* separated by an intron (dashed line), and the *ie1* ORF is only composed of *exon2*. The *ac146* ORF (gray arrow) is upstream of the *ie1* ORF and the late promoter for *ac146* resides within the *ie1* ORF. The knockout bacmid AcBac$^{ac146\text{-}ie1KO}$ was made from AcMNPV E2 bacmid (bMON14272) and the *ac146-ie1* locus replaced by recombination with the EM7 promoter-*zeocin* resistance gene (striped arrow) which deletes *ac146*, *ie1* and *ie0*. The knockout bacmid AcBac$^{ac146\text{-}ie1KO}$ was repaired by transposition to express *ie1, ie0*^{MtoA} or *ie0* under control of the *gp64* promoter (*gp64p*). Included in the repair vectors were two transposition sites (Tn7R and Tn7L), the gentamicin resistance gene (*gen*^{R}), green fluorescence protein gene (*gfp*), the polyhedrin gene (*polh*) and *ac146* (gray arrow) or *ac146HA* (not shown). The viral bacmids were named as follows; v*gp64*p-IE1, v*gp64*p-IE0^{MtoA}, and v*gp64*p-IE0. (B) Western blot time course analysis of IE0 and IE1 in v*gp64*p-IE1, v*gp64*p-IE0^{MtoA}, v*gp64*p-IE0 and wildtype (WT) infected cells. *Sf*9 cells were infected at an MOI of 5 and cells and supernatants were collected at 3, 6, 9, 12, 20, 24, 36 and 48 hpi. Whole cell lysates were separated by 10% SDS-PAGE and transferred to membranes. IE0 and IE1 were immunodetected with an anti-IE1 mouse monoclonal primary antibody. (C) Comparison of expression levels of IE1 and IE0 from v*gp64*p-IE1, v*gp64*p-IE0^{MtoA}, v*gp64*p-IE0 and wildtype (WT) infected *Sf*9 cells at 9 hpi. Actin was detected to show protein loading levels.

DNA replication (0–9 hpi), IE1 from v*gp64p*-IE1 appeared to be expressed at slightly higher levels than IE0 or IE1 from v*gp64p*-IE0 and v*gp64p*-IE0^{MtoA} and this was confirmed by direct comparison of the 9 hpi sample (Fig. 1C). All three of the viruses that utilized the *gp64* promoter expressed IE0 or IE1 at levels lower than in a wildtype infection (Fig. 1B and C).

Time course analysis of BV production supported by IE0 and IE1 in infected cells

In time course assays, we used v*gp64p*-IE1, v*gp64p*-IE0^{MtoA} and v*gp64p*-IE0 to compare the ability of IE0 and IE1 to support BV production in infected cells (Fig. 2A). In cells expressing IE0 or IE1 alone (v*gp64*p-IE0^{MtoA} and v*gp64*p-IE1) BV production was delayed in comparison to wildtype virus. BV production of wildtype virus initiated between 18 and 20 h while that of v*gp64*p-IE0^{MtoA} and v*gp64*p-IE1initiated between 20 and 24 hpi. In addition, BV levels of v*gp64*p-IE0^{MtoA} and v*gp64*p-IE1 increased only 100 fold by 48 hpi. However, no substantial difference was observed between IE0 and IE1 indicating that both proteins are equally efficient in supporting BV production. In cells expressing both IE0 and IE1 (v*gp64*p-IE0) BV production initiated normally at 18 hpi, and levels increased over 1000-fold by 48 hpi but did not increase to the same levels as that of wildtype infected cells. Thus, expression of IE0 and IE1 together, resulted in BV production initiating earlier and reaching higher levels than viruses expressing only IE0 or IE1.

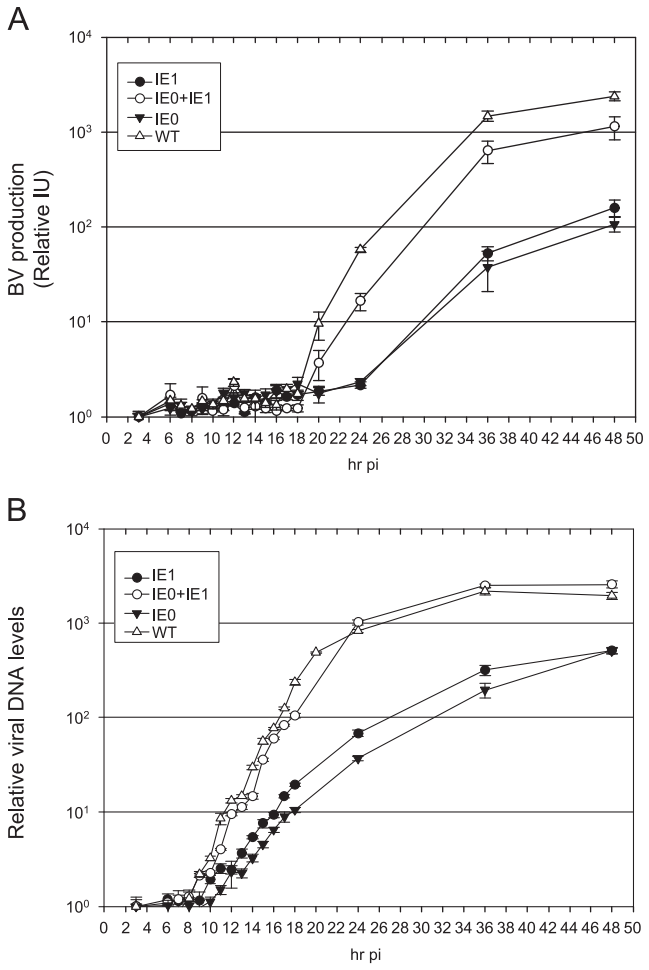

Fig. 2. Time course analysis of BV production and viral DNA replication in cells infected with viruses expressing IE1, IE0+IE1, or IE0, under control of the *gp64* promoter. BV production (A) or viral DNA levels (B) were determined from *Sf*9 cells infected with viruses expressing IE1 (v*gp64*p-IE1) (•), IE0+IE1 (v*gp64*p-IE0) (○), IE0 (v*gp64*p-IE0^{MtoA}) (▾) and wildtype (WT) (Δ). *Sf*9 cells were infected at an MOI of 5 and cell pellets were collected at hourly time points between 3 and 18 hpi, as well as 20, 24, 36 and 48 hpi. BV production and viral DNA levels were assessed by qPCR and each data point represents the average of 2 to 4 assays. The level of BV production or levels of DNA replication were normalized relative to 3 hpi for each virus and error bars represent standard error. IU, infectious units.

Time course analysis of viral DNA replication supported by IE0 and IE1 in BV infected cells

Levels of viral DNA replication during infection with BV were compared using qPCR to detect the number of genomes present in the harvested cell pellets at each time point. Time course data for each virus was normalized to 3 hpi (which represents the level of unreplicated input DNA), to reflect the increase in viral genomic DNA (Fig. 2B). DNA replication levels in cells expressing only IE0 (v*gp64*p-IE0^{MtoA}) or only IE1 (v*gp64*p-IE1) showed very similar profiles. Both of those viruses had delayed initiation of DNA replication relative to wildtype, and in addition, both viruses had similar increases in DNA levels which were approximately 500-fold by 48 hpi. No substantial differences therefore were observed between IE0 and IE1 in the ability to support DNA replication in virus infected cells. Interestingly however, the virus v*gp64*p-IE0, expressing both IE0 and low levels of IE1 (Fig. 1B, vgp64p-IE0), initiated DNA replication earlier than either virus expressing IE0 or IE1 alone. In addition, expression of IE0 plus IE1 resulted in over a 1000 fold increase in viral DNA levels by 48 hpi which was equivalent to wildtype virus (Fig. 2B, IE0+IE1). These results show that the virus expressing both IE0 and low levels of IE1 generated higher levels of viral DNA, than viruses expressing either IE0 or IE1 alone.

Transient viral DNA replication analysis using plasmids expressing equal levels of IE0 and IE1

The results of the analysis of virus replication in BV infected cells suggest that IE0 and IE1 were equivalent in their ability to support viral DNA replication. The assays using BV reflect the ability of IE0 or IE1 to support DNA replication in the context of infected cells expressing all other viral gene products. To further compare the ability of IE0 and IE1 to support viral DNA replication, transient DNA replication assays were used (Fig. 3). This approach allows IE0 and IE1 to be analyzed independently and only in the presence of the known essential viral replication factors that form the viral replication complex. The transient assays used a viral DNA replication reporter construct which contained the *hr5* replication origin and plasmids expressing the essential and stimulatory viral replication factors, LEF1, LEF2, LEF3, LEF7, IE2, DNAPOL HELICASE and P35 (Ahrens et al., 1995) and plasmids expressing IE0 or IE1 under control of the *gp64* promoter (p*gp64*p-IE0^{MtoA} and p*gp64*p-IE1) (Fig. 3A) identical to the promoter constructs used in the viruses (Fig. 1A). In addition, we constructed two additional plasmids that expressed IE0 or IE1 under control of the AcMNPV *ie1* promoter (p*ie1*p-IE0^{MtoA} and p*ie1*p-IE1) (Fig. 3A). Both sets of plasmids expressed equal levels of IE0 and IE1 under the same promoter but the *ie1* promoter plasmids expressed both proteins at about 1000 fold higher levels (Fig. 3B).

The levels of transient replication of the *hr5* replication origin plasmid were initially compared by Southern blot (Fig. 3C). No differences in the levels of DNA replication were observed when cells were co-transfected with plasmids expressing IE0 or IE1 under control of the *gp64* or *ie1* promoter. To further compare and quantify the amount of replicated *hr5* plasmid supported by equal levels of IE0 and IE1 transient replication assays were repeated and measured using qPCR (Fig. 3D). All transient assays with IE0 or IE1 whether expressed at low or high levels resulted in greater than 10^4 copies of the *hr5* plasmid. No significant difference therefore was detected between the abilities of IE0 and IE1 to support transient DNA replication.

Our analysis of viral DNA replication by transient replication assays suggests that IE0 and IE1 are equally efficient in their ability to support viral DNA replication. However, in the context of AcMNPV infection, replication of viral DNA and BV production

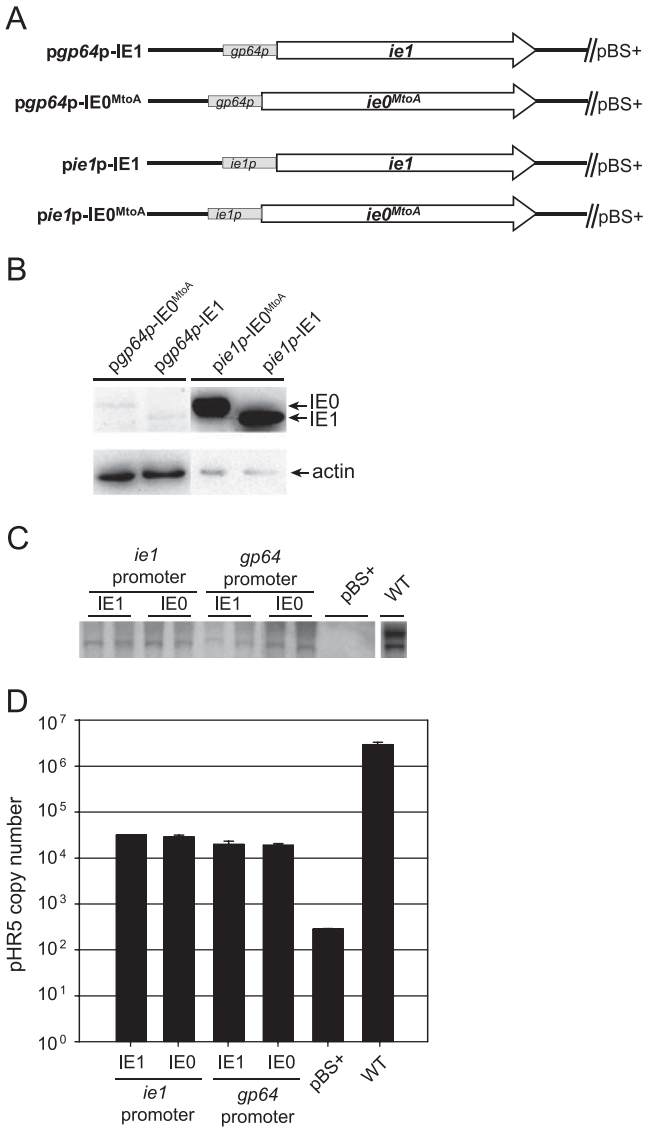

Fig. 3. Comparison of IE0 and IE1 support of transient DNA replication of a *hr5* origin containing plasmid measured by Southern blot and qPCR. (A) Schematic diagrams showing the plasmid constructs p*gp64*p-IE1 or p*gp64*p-IE0^{MtoA} and p*ie1*p-IE1 or p*ie1*p-IE0^{MtoA} used to express IE1 or IE0. (B) Western blot analysis showing equal levels of expression of IE0 and IE1 between the *gp64* promoter constructs or between the *ie1* promoter constructs shown in A. Lanes were loaded with 100,000 cell equivalents for p*gp64*p-IE1 or p*gp64*p-IE0^{MtoA} and 10,000 cell equivalents for p*ie1*p-IE1 or p*ie1*p-IE0^{MtoA}. IE0 and IE1 were immunodetected with an anti-IE1 mouse monoclonal primary antibody. Actin was immunodetected to show protein loading levels. (C) Southern blot of replicated *hr5* target plasmid DNA. *Sf*9 cells were transfected with a target plasmid containing the origin of replication *hr5* (1 μg), accompanied by the plasmids containing the replication factors *lef1*, *lef2*, *lef3*, *DNApol*, *helicase*, *pe38*, *ie2*, *lef7* and *p35* (0.5 μg of each), as well as a plasmid expressing IE1 or IE0 shown in A. Cells transfected with, the target plasmid and the plasmid vector pBS+ was a negative control, or AcMNPV wildtype (WT) bacmid DNA was a replication positive control. Replicated target plasmid was detected by hybridization to a ^{32}P-labeled *Hind*III-*Pst*I fragment of the *hr5* target plasmid. The replicated plasmid was visualized using a phosphoimager (Perkin-Elmer) and a representative blot is shown. (D) Detection of replicated *hr5* target plasmid DNA by qPCR. Total DNA (20 ng) from repeat transfections of plasmids described above were digested with 10U *Dpn*I. 5 ng of the digested total DNA was used as template for qPCR analysis. Values reported are an average of duplicate samples each with a technical replicate. Error bars represent standard error.

were higher when both IE0 and IE1 were present when compared with IE0 or IE1 alone. Combined, this would suggest that there may be different interactions between IE0 and IE1, and other viral factors, and that such interactions affect viral replication.

IE0 preferentially transactivates specific viral early gene promoters

If IE0 and IE1 are similar in their ability to support viral DNA replication, yet differ in their capacity to support overall viral replication, the functional difference between IE0 and IE1 may lie in their functions as potent viral transcriptional transactivators (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, 1993; Pathakamuri and Theilmann, 2002; Slack and Blissard, 1997; Taggart et al., 2012). To determine whether IE0 and IE1 are similar in their ability to transcriptionally transactivate viral early gene promoters, they were compared using transient transactivation studies. Plasmid p*gp64*p-IE0^{MtoA} or p*gp64*p-IE1 was used to express IE0 or IE1. Using mRNA isolated from cells infected with AcMNPV expressing only IE0 or IE1 for screening with a previously described microarray of the AcMNPV genome (Yamagishi et al., 2007), we identified a large number of candidate viral genes that were potentially differentially activated by IE0 or IE1. Based on that analysis, transient transactivation assays were performed with 19 *cat* reporter constructs containing the promoters of *lef4*, *lef6*, *ac79*, *p35*, *ac18*, *lef3*, *ac111*, *39K*, *ie1*, *ie2*, *pe38*, *me53*, *ie0*, *gp64*, *p78*, *ac33*, *ac91*, *ac52* and *ac76* (Fig. 4A).

The results from the transient transactivation assays are presented as CPM comparing absolute transactivation levels (Fig. 4B upper panel) or normalized relative to IE1 transactivation levels to compare directly the activation levels of IE0 and IE1 (Fig. 4B lower panel). The 19 viral early gene promoters examined had a range of responses to IE0 and IE1 transactivation but in general any promoter that was transactivated by IE0 was also transactivated by IE1. Thus, we did not identify any promoter that was only transactivated by IE0 or IE1. However, eight promoters, *lef4*, *lef6*, *ac79*, *p35*, *ac18*, *lef3*, *ac111* and *39K* were differentially regulated, and transactivated to higher levels by IE0 than by IE1. These eight promoters had between 3 and 8 fold higher levels of expression when transactivated by IE0 compared to IE1. These promoters therefore appear to be substantially more sensitive to transactivation by IE0.

A second group of early promoters that included *ie1*, *ie2*, *pe38*, *me53*, *ie0* and *gp64* was constitutively expressed at significantly higher levels and was unaffected positively or negatively by either IE0 or IE1 transactivation (Fig. 4). In addition, five other promoters (Fig. 4; *p78*, *ac33*, *ac91*, *ac52* and *ac76*) had expression levels that were at or slightly above background. Only minor or no increase was observed when transactivated by IE0 or IE1. Thus, under these conditions we detected little or no transcriptional activity from these viral promoters and no transactivation by IE0 or IE1.

The higher levels of transactivation observed for *lef4*, *lef6*, *ac79*, *p35*, *ac18*, *lef3*, *ac111* and *39K* promoters represents the first report of a quantitative functional difference between IE0 and IE1. To further investigate this observation, a more extensive transactivation analysis of two promoters (*lef3* and *39K)* was performed. Each was analyzed in the presence of increasing amounts of transactivator plasmid expressing IE0 or IE1, under the control of either the *gp64* (p*gp64*p-IE1 or p*gp64*p-IE0^{MtoA}) or *ie1* promoter (p*ie1*p-IE1 or p*ie1*p-IE0^{MtoA}) (Fig. 5A and B). The use of the two sets of plasmids permits the comparison of IE0 and IE1 transactivation under relatively low or high cellular expression levels as the *ie1* promoter expresses IE0 or IE1 at higher levels than the *gp64* promoter as shown in Fig. 3B. When the transactivators were expressed at low levels (under control of the *gp64* promoter), both *lef3* and *p39* promoters were transactivated to higher levels by IE0 than by IE1 (5A). The levels of transactivation by IE0 were approximately 0.5 to nearly 5 fold higher than IE1 for *lef3* and 2 to nearly 10 fold higher for *39K*, over the range of plasmid concentrations. When IE0 and IE1 were expressed at much higher

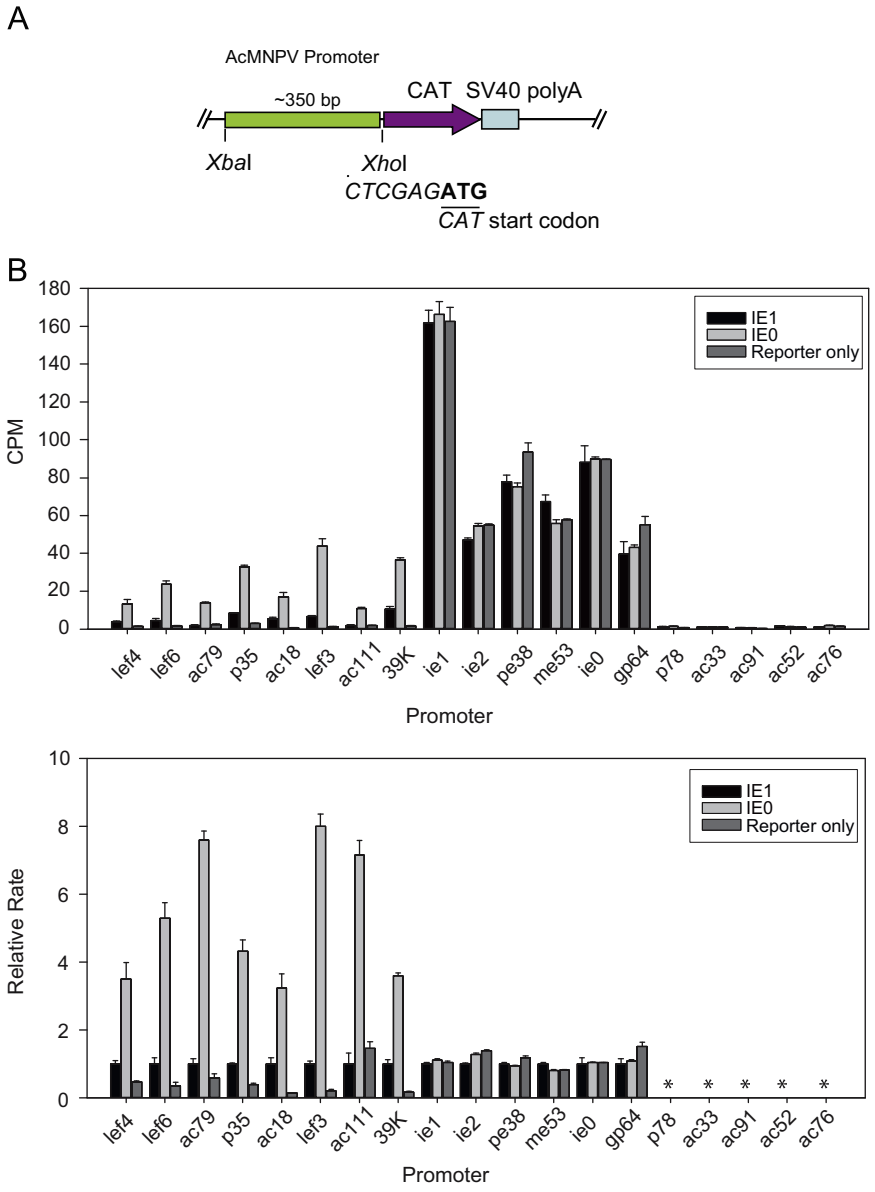

Fig. 4. Transactivation analysis of AcMNPV early gene promoters by IE0 and IE1. (A) Schematic diagram showing the general construction of 19 early gene CAT reporter constrcts. (B) *Sf*9 cells were co-transfected with 0.5 μg of 19 viral early gene promoter-CAT constructs (indicated on the *x*-axis), and 0.5 μg of the transactivator plasmids expressing IE1 or IE0 under control of the *gp64* promoter (p*gp64*p-IE1 or p*gp64*p-IE0^{MtoA}). Top panel shows the observed CPM comparing absolute transactivation levels. Panel B shows transactivation results normalized relative to IE1 transactivation levels to directly compare the activities of IE0 and IE1 on each promoter regardless of level of expression. CAT assays were performed as described in the *Materials and methods* and error bars represent standard error. Asterisks represent normalized samples that had no significant expression relative to background.

levels (under the control of the *ie1* promoter) we observed no significant differences in the levels of transactivation for *lef3* at all plasmid concentrations (Fig. 5B). In parallel, the *39K* promoter was transactivated by IE0 at approximately 2–3 fold higher levels at the lowest plasmid concentrations, but as the IE0 levels increased no differences were observed (Fig. 5B). These more detailed analyses of the *lef3* and *39k* promoters are consistent with previous results (Fig. 4); showing for the first time that specific viral promoters can be significantly more responsive to IE0 than to IE1. However, we found that this functional difference was only observed when cellular concentrations of the transactivators were low.

Discussion

The replication cycle of the archetype alphabaculovirus AcMNPV is dependent upon the production of the two essential transregulatory proteins IE0 and IE1 (Stewart et al., 2005). The two proteins are identical except for the N-terminal 54 amino acids of IE0 which is a result of one of the rare baculovirus splicing events (Chen et al., 2013; Chisholm and Henner, 1988). IE0 expression peaks very early in infection prior to the initiation of viral DNA replication whereas IE1 levels continue to increase throughout infection, resulting in a varying ratio of IE0 to IE1 throughout the viral life cycle (Huijskens et al., 2004; Kremer and Knebel-Morsdorf, 1998; Lu et al., 2003; Stewart et al., 2005). The goal of this study was to determine the separate functional roles of AcMNPV IE0 and IE1 during the virus infection cycle. Earlier studies have shown that if a virus expresses only IE0 or IE1, the virus is able to replicate, producing both ODV and BV, but significant differences were evident in BV production and levels of DNA replication. This showed that both IE0 and IE1 can support virus replication but suggests they have different roles in either viral DNA replication or transcriptional transactivation that may be

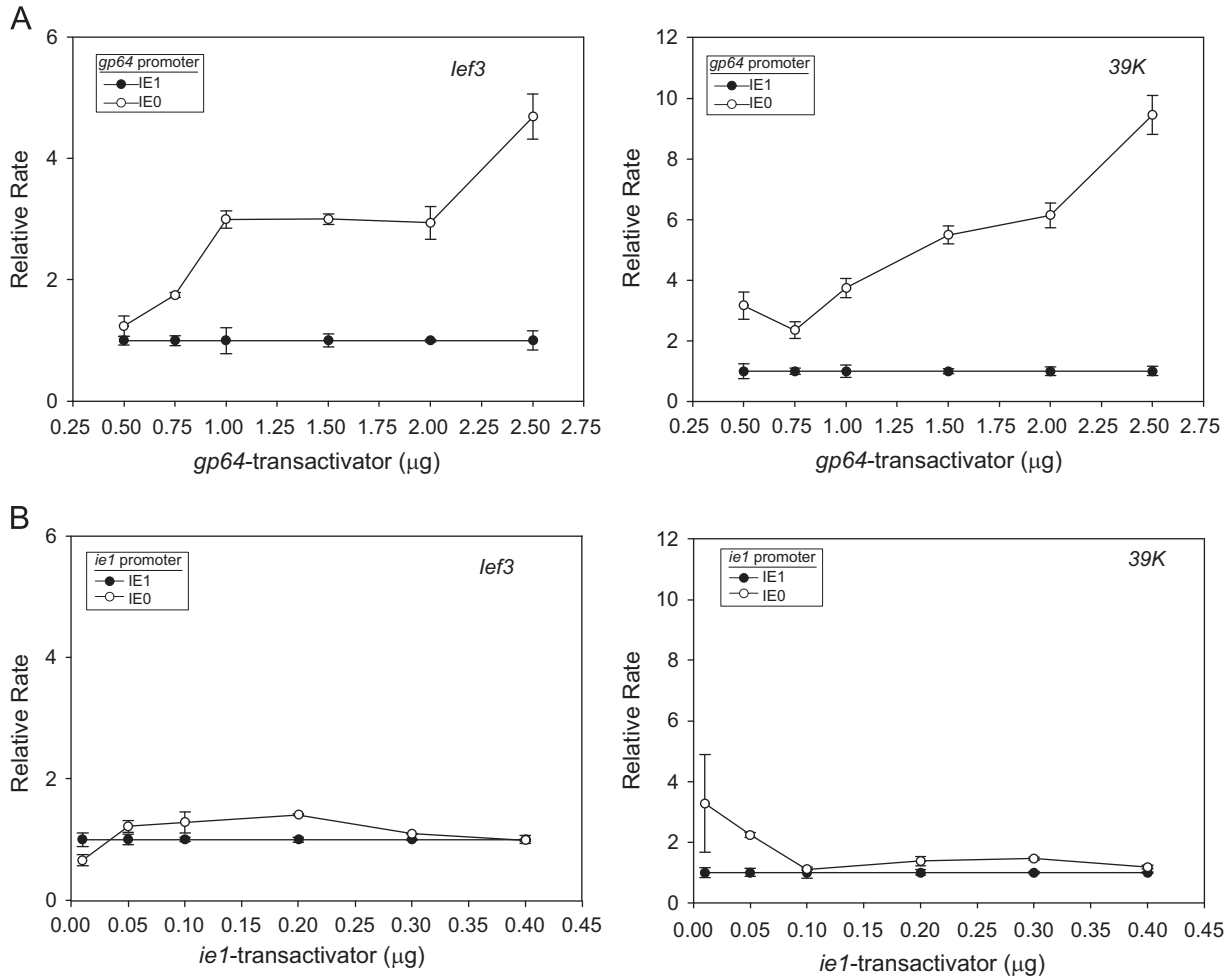

Fig. 5. Transactivation of *lef3*CAT and *39K*CAT by increasing amounts of IE0 or IE1. *Sf*9 cells were co-transfected with 0.5 μg of p*lef3*CAT or p*39K*CAT and either (A) 0 0.5, 0.75, 1, 1.5, 2, or 2.5 μg of pg*p64*p-*ie1* (IE1, •) or pg*p64*p-*ie0*^{MtoA} (IE0,○) or (B) 0.01, 0.05, 0.1, 0.2, 0.3, or 0.4 μg of p*ie1*p-*ie1* (IE1,•) or p*ie1*p-*ie0*^{MtoA} (IE0,○). All samples were normalized relative to IE1 transactivation levels. Error bars represent standard error.

dependent on time of expression or the levels of expression (Stewart et al., 2005). In prior studies that examined IE0 and IE1 function, both proteins were expressed under the control of their native *ie0* and *ie1* promoters (Nie, 2010; Stewart et al., 2005), which results in different temporal expression and different levels and could account for the observed differences. To address this issue in the current study, we used an alternate approach in which recombinant viruses or plasmids were constructed that used identical promoters to drive expression *ie0* or *ie1* in infected cells or in transient assays. Our results indicate that IE0 and IE1 are equivalent in their ability to initiate and support viral DNA replication and BV production, but differentially transactivate specific viral genes.

The presence of both IE0 and IE1 ensure efficient viral DNA replication, but neither offers a functional advantage in the replisome complex when expressed separately

As indicated above prior research had suggested that IE1 was potentially more efficient for DNA replication compared to IE0 since virus producing only IE1 had higher levels of viral DNA in infected cells (Stewart et al., 2005). In contrast, the results of this study show that in virus infected cells if IE0 or IE1 are expressed at similar levels and times by using the same promoter no significant difference between the time of initiation and the level of DNA replication was observed (Fig. 2B). Transient DNA replication assays also showed no difference in ability of IE0 or IE1 to replicate viral DNA (Fig. 3). This suggests that there may be no difference in the function of IE0 and IE1 in the viral replisome complex consisting of the essential and stimulatory viral replication factors LEF1, LEF2, LEF3, DNA polymerase, helicase, P35 and IE2. However, surprising results were observed in cells infected with the virus v*gp64*-IE0 (Fig. 2B), which expresses both IE0 and lesser amounts of IE1, viral DNA replication is initiated earlier and reached higher final levels when compared to cells infected with viruses expressing only IE0 or only IE1. These results clearly suggest that there is a synergistic interaction between these two proteins that results in enhanced and more rapid viral DNA replication compared to either protein by itself. It would therefore appear there is a distinct advantage for expressing both IE0 and IE1 as it results in more potent stimulation of viral DNA replication compared to expression of each protein separately.

When levels are limiting IE0 transactivates select viral early promoters to higher levels

The analysis of viral DNA replication indicated that IE0 and IE1 appear to be functionally identical. This suggested that differences between IE0 and IE1 would be in their other core role of transcriptional activation. All promoters analyzed to date that have been shown to be transactivated by IE1 are also transactivated by IE0 but levels of activation have varied significantly

(Huijskens et al., 2004; Kovacs et al., 1991; Nie, 2010). However, in these past studies the level of expression of each transactivator was not equalized and could have potentially masked differences in function. Using transient gene expression assays, we examined 19 viral promoters for their ability to be transactivated by IE0 or IE1. IE0 or IE1 was produced from plasmids expressing the genes under control of the identical *gp64* promoter and in addition the *ie1* promoter. Using this approach differences in transactivator function were readily observed. Eight viral early gene promoters were transactivated *(lef4, lef6, ac79, p35, ac18, lef3, ac111,* and *39K*) to a higher level by IE0 than with IE1 (Fig. 4). These results are the first study to show that IE0 can be a more potent activator of specific promoters strongly suggesting that IE0 and IE1 can provide differential transcriptional regulation of viral genes. Additional results show that if IE0 and IE1 are expressed at relatively high levels the differences in gene activation are not observed. Therefore the functional differences between IE0 and IE1 are dependent on cellular concentration. This was confirmed by transactivation analysis of *lef3* and *39K* with varying levels of transactivators under control of the *gp64* or the significantly stronger *ie1* promoter (Fig. 5). Both *lef3* and *39K* promoters were significantly more sensitive to IE0 than IE1 at low levels of expression. However, when IE0 and IE1 reached high enough levels (under control of the *ie1* promoter), no difference was observed between IE0 and IE1. These results show that specific viral promoters are more responsive to IE0 than to IE1, when the cellular levels of the transactivators are low.

These results appear to be reflective of the biology of the AcMNPV infection at early times post-infection. Immediately upon infection with BV during the first 6 h in cultured cells, IE0 is naturally expressed at higher levels than IE1 but overall total cellular levels are low (Huijskens et al., 2004). Based on our results IE0 responsive genes would be more highly expressed during this very early period of infection. In virus infected cells expression of IE0 with low levels of IE1 initiated viral DNA replication faster and to higher levels than IE0 alone (Fig. 3B). This would suggest that IE0 plus a low level of IE1 is optimal for viral DNA replication when both proteins are limiting. These results could also explain why the spliced *ie0* mRNA is translated as both proteins as it would ensure efficient viral DNA replication when levels of IE0 and IE1 are low which may be essential to ensure rapid budded virus production. Interestingly, two of the IE0 sensitive genes, *lef3* and *p35*, regulate genes that are required for or augment viral DNA replication (Kool et al., 1994a). In addition, *p35* is essential for inhibiting the host defensive apoptosis response (Clem and Miller, 1993). Of the other genes that are more responsive to IE0 transactivation, *lef4, lef6* and *39K* are required for late gene expression (Todd et al., 1995), *ac79* impacts BV virus production and *ac18* deletion viruses take longer to kill the host (Wang et al., 2007; Wu and Passarelli, 2012), whereas *ac111* has no known function (Ono et al., 2012). Low or high levels of IE0 or IE1 expression did not have any impact on 11 of the 19 promoters analyzed; therefore they would presumably be unaffected directly but could be indirectly regulated by the varying levels of these transregulatory proteins during a normal virus infection. Previous analyses of *Lymantria dispar* MNPV (LdMNPV) *ie0* and *ie1* suggested that only *ie0* was able to transactivate the AcMNPV *p39* promoter (Pearson and Rohrmann, 1997). This may be similar to the results in this study which showed that *p39* promoter was more responsive to low levels of IE0; however the expression levels of IE0 and IE1 in that LdMNPV study were unknown.

Alphabaculoviruses are the only genera of *Baculoviridae* to encode *ie0* homologs, the only spliced baculovirus gene that is known to produce multiple protein products, though more spliced genes have recently been discovered (Chen et al., 2013). The acquisition of *ie0* by the alphabaculoviruses would support the idea that IE0 and IE1 have added to the success of alphabaculoviruses, perhaps by enhancing the speed of viral replication. There is strong evidence that upon infection of lepidopteran larvae, alphabaculoviruses attempt to rapidly escape the midgut to avoid the host defense of cell sloughing (Granados and Lawler, 1981; Haas-Stapleton et al., 2003; Milks et al., 2003; Washburn et al., 2002; Washburn et al., 2003). In addition, it has been documented that occlusion bodies are not produced or greatly reduced in midgut cells (Bonning, 2005; Granados and Lawler, 1981; Harrap and Robertson, 1968) and interestingly co-expression of IE0 with IE1 was antagonistic for very late polyhedrin gene expression (Huijskens et al., 2004). These data suggest that specifically in the midgut, alphabaculoviruses may favor BV production over ODV for at least the early times post-infection, to ensure midgut escape and enhancing efficient systemic infection. The results of this study therefore support the conclusion that the function of IE0 is specifically during the first hours of midgut infection when viral protein levels are low. Selective enhanced transactivation of specific viral genes may facilitate more rapid viral DNA replication and production of BV to enable increased success of establishing a systemic infection of the host organism.

Materials and methods

Cells and viruses

*Sf*9 cells were maintained at 27 °C 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 strain E2 (Invitrogen Life Technologies) was used to construct all AcMNPV recombinant bacmids in *Escherichia coli* (*E. coli*) strain DH10B as described previously (Datsenko and Wanner, 2000; Luckow et al., 1993). Wildtype virus is designated as bMON14272 expressing polyhedrin and GFP as previously described (Stewart et al., 2005).

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

The AcMNPV *ie1* promoter was amplified with primers 1932 and 1931 using pAcIE1-DT1 (Theilmann and Stewart, 1991) as template and cloned into pBS+ to generate p*ie1*p. The AcMNPV *gp64* promoter was amplified with primers 1933 and 1934 using AcMNPV virus strain E2 as template and cloned into pBS+ to generate p*gp64*p. The amplified *gp64* promoter contained 350 bp upstream from the *gp64* ATG start codon. The ORFs for *ie0, ie0^{MtoA}* or *ie1* were amplified and inserted into both p*ie1*p and p*gp64*p produced the plasmids 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. Each ORF was amplified with a common 3′ primer 1937, which contained the OpMNPV *ie2* polyA signal. Both *ie0^{MtoA}* and *ie0* were amplified with a common 5′ primer 1939. The *ie1* ORF was amplified using the 5′ primer 1938. 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. The sequences of the primers used in this study are listed in Table S1.

Construction of bacmid transfer plasmids

Two pFAcT-GFP transfer plasmids (Dai et al., 2004) were constructed to contain the *ac146* ORF and a 3′ hemagglutinin (HA)-epitope tagged *ac146* ORF (*ac146HA*) to produce plasmids pF and pF$_{HA}$. The *ac146* ORF was amplified using primers 1936 and

1935 and AcMNPV virus strain E2 was used as template. Primer 1936 contained the heterologous OpMNPV *op146* late promoter and primer 1935 contained the OpMNPV *ie2* polyA signal. The OpMNPV *op146* late promoter was used to reduce potential intragenomic homologous recombination. The *ac146HA* ORF was amplified using primers 1936 and 1940 and AcMNPV virus strain E2 was used as template. Primer 1940 contained the OpMNPV *ie2* polyA signal and sequence coding for the HA-epitope. The cassettes from plasmids p*gp64*p-IE0, p*gp64*p-IE0^{MtoA} and p*gp64*p-IE1 were removed and cloned into pF and pF$_{HA}$ producing six transfer plasmids.

Construction of ac146-ie1 knockout and repair viruses

An *ie0, ie1* and *ac146* KO mutant was made by replacing the *ac146-ie1* coding sequence with an EM7-*zeocin* resistance cassette in the AcMNPV bacmid bMON14272 (Fig. 1). The EM7-*zeocin* resistance cassette was amplified using primers 1551 and 1918 and the plasmid p2Zop2E (Pfeifer et al., 1997) was used as template. Primer 1551 incorporated the flanking homologous region of the 3′ end of *ac145*, a polyA signal for *ac145* and a region homologous to the EM7 promoter-*zeocin* cassette. Primer 1918 incorporated the 3′ flanking region of *ie1*, a polyA signal for *pif5* and a region homologous to the EM7 promoter-*zeocin* cassette. The amplified EM7 promoter-*zeocin* resistance cassette was transformed into competent *E. coli* BW25113 cells containing bMON14272 and recombinase helper plasmid (pKD46) (Datsenko and Wanner, 2000). A positive colony named AcBac$^{ac146\text{-}ie1KO}$ was selected for kanamycin and zeocin resistance and the deletion of *ac146-ie1* was confirmed by PCR using primers 1574 and 520, and 1919 and 1920. AcBac$^{ac146\text{-}ie1KO}$ was transformed into electrocompetent *E. coli* DH10B cells containing the transposase helper plasmid (pMON7124) (Luckow et al., 1993). Six repair bacmids were made by transforming the DH10B cells containing AcBac$^{ac146\text{-}ie1KO}$ and pMON7124 with each transfer plasmid.

To produce budded virus stocks, *Sf*9 cells were transfected using lipofectin with 1 μg of each repair bacmid as previously described (Campbell, 1995). BV supernatants were harvested at 9 dpt and were used to infect 2.5×10^7 cells at an estimated multiplicity of infection (MOI) of 0.6. After 5 days at 27 °C, the BV supernatants were collected. Virus stocks were named, v*gp64*p-IE1, v*gp64*p-IE0, v*gp64*p-IE0^{MtoA}, v$_{HA}$*gp64*p-IE1, v$_{HA}$*gp64*p-IE0, and v$_{HA}$*gp64*p-IE0^{MtoA}. The "$_{HA}$" denotes the virus with AC146 tagged with the HA epitope at the C-terminus. TCID$_{50}$ end point dilution was performed in duplicate to estimate viral titre by infecting *Sf*9 cells in 96-well microtitre plates (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 titers were calculated using Chiptitre software (Lynn, 1992).

Plasmids used for transient viral replication assays

Plasmids containing *lef1*, *lef2*, *lef3*, *DNApol*, *helicase*, *lef7*, and *ie2* were kindly provided by Dr. Lorena Passarelli (Kansas State University). These seven plasmids contained one of the respective ORF's under control of the *Drosophila melanogaster* heat shock protein 70 promoter and carried an N-terminal HA-tag within the pHSEpiHis backbone (Rapp et al., 1998). A plasmid containing *p35* within the plasmid backbone p2Zop2E (Pfeifer et al., 1997) was generated by amplifying the *p35* ORF using primers p355d3 and p353*Xho* using AcMNPV virus strain E2 as template. The target replication plasmid pAchr5-CAT contained the viral origin of replication *hr5* within the pie1-Bgal-cat-pA backbone (Nie, 2010).

Construction of viral early gene promoter-chloramphenicol acetyl-transferase (CAT) reporter plasmids

Nineteen viral early gene promoters *lef4, lef6, p35, ac79, p35, ac18, lef3, ac111, 39K, ie1, pe38, me53, ie0, gp64, p78, ac33, ac91, ac52,* and *ac76* were amplified by PCR with primers listed in Supplementary Table S1. The promoters were cloned into the CAT expression vector p*ie1*-Bgal-cat-pA backbone containing a multiple cloning site upstream of the *cat* ORF and the SV40 polyadenylation signal sequence (Fig. 4A). The promoter regions utilized were all approximately 350 bp upstream from the native translational start site and were cloned 6 bp upstream from the *cat* gene start codon.

Insect cell transfections

*Sf*9 cells cultured in TNM-FH media were dispensed into 6-well plates at 1×10^6 cells per well. Lipofectin was used to transfect plasmid constructs as previously described (Campbell, 1995). The cells were overlaid with the lipofectin–DNA mixture and incubated at 27 °C for 4 h. After incubation, the lipofectin–DNA 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. All transfections were performed in duplicate.

In transient replication assays, 1.0 μg of the *hr5* target replication plasmid, 0.5 μg of each replication factor, and 0.5 μg of empty plasmid (pBS+) or p*gp64*p-IE0, p*gp64*p-IE0^{MtoA} and p*gp64*p-IE1 were co-transfected into *Sf*9 cells. Co-transfection of 0.5 μg of wildtype AcBac and 1.0 μg of the *hr5* target replication plasmid acted as a positive replication control. For Western blots comparing expression of IE0 and IE1 expressed from the plasmids cells were transfected with 10 μg plasmid DNA.

Transient transactivation assays were performed by transfecting 0.5 μg of a viral early gene promoter-CAT reporter plasmid and 0.5 μg of p*gp64*p-IE0, p*gp64*p-IE0^{MtoA} or p*gp64*p-IE1 into *Sf*9 cells. In titration of transactivator assays (Fig. 5), 0.5 μg of a viral early gene promoter-CAT reporter plasmid and 0.5, 0.75, 1, 1.5, 2, or 2.5 μg of p*gp64*p-IE0^{MtoA} or p*gp64*p-IE1, or 0.01, 0.05, 0.1, 0.2, 0.3, or 0.4 μg of p*ie1*p-IE0^{MtoA} or p*ie1*p-IE1 were co-transfected into *Sf*9 cells. The lipofectin/Grace's media ratio was determined for each assay by titration. The total amount of plasmid transfected for each assay was equalized by addition of pBS+ plasmid.

Time course infection assays

Two time course infection assays were performed in duplicate with some variation as noted. For both assays, *Sf*9 cells (3×10^6 cell/50 ml tube) were infected with v*gp64*p-IE1, v*gp64*p-IE0, v*gp64*p-IE0^{MtoA} and WT, at an MOI of 5 at 27 °C for 1 h. The cells were washed with Grace's insect media and gently resuspended in TNM-FH media. In one assay, the cells were resuspended for a final concentration of 3×10^5 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. In the second assay resuspended cells were immediately dispensed into micro test tubes at 1.5×10^5 cells/0.5ml. Micro test tube corresponding to each virus were removed at 3 hpi, at hourly time points between 6 to 18 hpi, and 24, 36, and 48 hpi. In both assays, each sample at each time point was centrifuged at 5000*g* for 5 min. BV supernatants were removed and cell pellets were washed (1× PBS) and either split for further use for Western analysis and DNA replication analysis or immediately resuspended in lysis buffer (10 mM TrisCl pH 8.0, 100 mM EDTA, 0.5% SDS).

Time course analysis of viral DNA replication and BV production

To analyze viral DNA replication and BV production, a previously described qPCR method was used (McCarthy and Theilmann, 2008; Vanarsdall et al., 2007). For DNA replication, frozen cell pellets (1.5×10^5 cells) from the first pair of time course infection assays were resuspended with a 0.4 M sodium hydroxide, 125 mM EDTA solution to dissolve occlusion bodies, and were incubated at 100 °C for 10 min. Cells were neutralized with 0.4M HCl and 5×10^4 cells were removed for analysis and treated with lysis buffer (10 mM TrisCl pH 8.0, 100 mM EDTA, 0.5% SDS). For the second pair of infectious time course assays previously treated with lysis buffer, 1.25×10^5 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. For all budded virus supernatants collected over the four time course assays, 100 μl 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. For DNA replication analysis, serial dilutions of previously quantified wildtype bacmid DNA were used as template for qPCR to construct a standard curve. For BV production analysis, serial dilutions of previously titred wildtype AcMNPV budded virus were used as template for qPCR to construct a standard curve. For all cell preparations, 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 × 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 s, 52 °C for 24 s, 72 °C for 30 s; followed by a melting curve analysis (McCarthy and Theilmann, 2008; Vanarsdall et al., 2007). Results were analyzed using MX4000 software (Stratagene). Technical replicates were performed for each qPCR reaction.

Southern blot analysis of transient DNA replication

Transient replication assays were performed as previously described (Ahrens et al., 1995). Cells from transient replication transfections were resuspended in lysis buffer (10 mM Tris pH 8, 1 mM EDTA, 0.2% SDS, 1 mg/ml proteinase K). Nucleic acid was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), followed by chloroform and treated with RNaseA (1 μg/μl). DNA was ethanol precipitated and resuspended in TE (10:1). Each DNA sample was quantified by running 1 μl on a 1% agarose gel with high mass ladder (Invitrogen) and analyzed using densitometry (ImageJ; NIH). DNA (2.5 μg) was linearized using *Pst*I (32U) and digested with *Dpn*I (8U) to remove unreplicated plasmid DNA. Digested DNA was run on a 2% agarose gel, transferred by alkaline blotting to a Zeta-probe (Bio-Rad) membrane. Membrane-bound digested DNA was hybridized to a *Hind*III-*Pst*I fragment of the *hr5* replication plasmid radio-labeled with [^{32}P]dCTP (random primer labeling kit, Invitrogen). Signal was visualized using PhosphoImager analysis (Perkin-Elmer). Cells transfected with the target plasmid and WT bacmid were used as a replication positive control.

Analysis of transient DNA replication by qPCR

DNA (20 ng) from transient replication transfections was digested with *Dpn*I (10U). In a 10 μl reaction volume, 5 ng of the digested DNA was used as template, with primers 2197 and 2198 (0.5 μM of each) and SsoFast Evagreen® Supermix (Bio-Rad). The qPCR thermal profile was; 1 cycle of 98 °C for 10 min; 40 cycles of 98 °C for 10 s, 55 °C for 15 s; 72 °C for 30 s; followed by a melting curve analysis. Technical replicates were performed for each qPCR reaction.

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 5 mM chloramphenicol, 210 mM TrisCl (pH 7.8), 125 μM acetyl coenzyme A and 0.014 μCi 3H-acetyl-CoA was added to 25 μl 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 (LS-6500, Beckman). To support results, replicated randomized block analysis of variance (ANOVA) followed by a TukeyHSD post-hoc test were performed. Statistical significance is defined as $p < 0.05$.

Western blot analysis

Cell pellets were passed through a 25 gauge syringe to shear genomic DNA and boiled to denature the protein samples. Equivalent numbers of cells were loaded onto each lane of 10% gels for sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE gels (Laemmli, 1970) were run using the Mini Protean II system (Bio-Rad) and protein was transferred by electroblotting at 100 V overnight to PVDF membranes (Millipore). Blots were probed with either primary mouse monoclonal anti-IE1 antibody (IE1-4B7) at 1:10,000 dilution (Ross and Gaurino, 1997) or primary mouse monoclonal anti-HA epitope antibody at 1:1000 dilution. Actin was detected using anti-actin antibody diluted 1:5000 (BD Biosciences cat#612657). Bound antibodies were detected using a secondary peroxidase conjugated goat anti-mouse antibody at 1:10,000 dilution. Blots were exposed and visualized with a Western-Lightening® Plus ECL Enhanced Chemiluminescence System (Perkin-Elmer).

Acknowledgments

The study was partially supported by grants to DAT from the Natural Sciences and Engineering Research Council of Canada and the Crop Genomics Initiative from Agriculture and Agri-Food Canada

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.07.044.

References

Ahrens, C.H., Carlson, C., Rohrmann, G.F., 1995. Identification, sequence, and transcriptional analysis of lef-3, a gene essential for *Orgyia pseudotsugata* baculovirus DNA replication. Virology 210 (2), 372–382.

Blissard, G.W., Rohrmann, G.F., 1991. Baculovirus gp64 gene expression: analysis of sequences modulating early transcription and transactivation by IE1. J. Virol. 65 (11), 5820–5827.

Bonning, B.C., 2005. Baculoviruses: biology, biochemistry, and molecular biology. In: Gilbert, L.I., Iatrou, K., Gill, S.S. (Eds.), Comprehensive Molecular Insect Science. Elsevier, Amsterdam.

Campbell, M.J., 1995. Lipofectin reagents prepared by a simple ethanol injection technique. Biotechniques 18 (6), 1027–1032.

Chen, Y.R., Zhong, S., Fei, Z., Hashimoto, Y., Xiang, J.Z., Zhang, S., Blissard, G.W., 2013. The transcriptome of the baculovirus *Autographa californica* multiple nucleopolyhedrovirus in *Trichoplusia ni* cells. J. Virol. 87 (11), 6391–6405.

Chisholm, G.E., Henner, D.J., 1988. Multiple early transcripts and splicing of the *Autographa californica* nuclear polyhedrosis virus IE-1 gene. J. Virol. 62 (9), 3193–3200.
Choi, J., Guarino, L.A., 1995a. The baculovirus transactivator IE1 binds to viral enhancer elements in the absence of insect cell factors. J. Virol. 69 (7), 4548–4551.
Choi, J., Guarino, L.A., 1995b. Expression of the IE1 transactivator of *Autographa californica* nuclear polyhedrosis virus during viral infection. Virology 209, 99–107.
Choi, J., Guarino, L.A., 1995c. A temperature-sensitive IE1 protein of *Autographa californica* nuclear polyhedrosis virus has altered transactivation and DNA binding activities. Virology 209, 90–98.
Clem, R.J., Miller, L.K., 1993. Apoptosis reduces both the in vitro replication and the in vivo infectivity of a baculovirus. J. Virol. 67 (7), 3730–3738.
Dai, X., Stewart, T.M., J.A., P., Li, Q., Theilmann, D.A., 2004. *Autographa californica* multiple nucleopolyhedrovirus *exon 0* (*orf141*), which encodes a RING finger protein, is required for efficient production of budded virus. J. Virol. 78 (18), 9633–9644.
Datsenko, K.A., Wanner, B.L., 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U. S. A. 97, 6640–6645.
Dickison, V.L., Willis, L.G., Sokal, N.R., Theilmann, D.A., 2012. Deletion of the AcMNPV ac146 eliminates the production of budded virus. Virology 431, 29–39.
Granados, R.R., Lawler, K.A., 1981. In vivo pathway of *Autographa californica* baculovirus invasion and infection. Virology 108 (2), 297–308.
Guarino, L.A., Summers, M.D., 1986a. Functional mapping of a *trans*-activating gene required for expression of a baculovirus delayed-early gene. J. Virol. 57 (2), 563–571.
Guarino, L.A., Summers, M.D., 1986b. Interspersed homologous DNA of *Autographa californica* nuclear polyhedrosis virus enhances delayed-early gene expression. J. Virol. 60 (1), 215–223.
Guarino, L.A., Summers, M.D., 1988. Functional mapping of *Autographa californica* nuclear polyhedrosis virus genes required for late gene expression. J. Virol. 62 (2), 463–471.
Haas-Stapleton, E.J., Washburn, J.O., Volkman, L.E., 2003. Pathogenesis of *Autographa californica* M nucleopolyhedrovirus in fifth instar *Spodoptera frugiperda*. J. Gen. Virol. 84 (Pt. 8), 2033–2040.
Harrap, K.A., Robertson, J.S., 1968. A possible infection pathway in the development of a nuclear polyhedrosis virus. J. Gen. Virol 3, 221–225.
Huijskens, I., Li, L., Willis, L.G., Theilmann, D.A., 2004. Role of AcMNPV IE0 in baculovirus very late gene expression. Virology 323, 120–130.
Kawasaki, Y., Matsumoto, S., Nagamine, T., 2004. Analysis of baculovirus IE1 in living cells: dynamics and spatial relationships to viral structural proteins. J. Gen. Virol. 85, 3575–3583.
Kool, M., Ahrens, C.H., Goldbach, R.W., Rohrmann, G.F., Vlak, J.M., 1994a. Identification of genes involved in DNA replication of the *Autographa californica* baculovirus. Proc. Natl. Acad. Sci. U. S. A. 91, 11212–11216.
Kool, M., Ahrens, C.H., Vlak, J.M., Rohrmann, G.F., 1995. Replication of baculovirus DNA. J. Gen. Virol. 76, 2103–2118.
Kool, M., Voeten, J.T.M., Goldbach, R.W., Vlak, L.M., 1994b. Functional mapping of regions of the *Autographa californica* nuclear polyhedrosis viral genome required for DNA replication. Virology 198, 680–689.
Kovacs, G.R., Guarino, L.A., Summers, M.D., 1991. Novel regulatory properties of the IE1 and IE0 transactivators encoded by the baculovirus *Autographa californica* multicapsid nuclear polyhedrosis virus. J. Virol. 65 (10), 5281–5288.
Kremer, A., Knebel-Morsdorf, D., 1998. The early baculovirus he65 promoter: on the mechanism of transcriptional activation by IE1. Virology 249, 336–351.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 (5259), 680–685.
Leisy, D.J., Rohrmann, G.F., 1993. Characterization of the replication of plasmids containing *hr* sequences in baculovirus-infected *Spodoptera frugiperda* cells. Virology 196, 9.
Lin, G., Blissard, G.W., 2002. Analysis of an *Autographa californica* nucleopolyhedrovirus lef-11 knockout: LEF-11 is essential for viral DNA replication. J. Virol. 76 (6), 2770–2779.
Lu, A., Carstens, E.B., 1993. Immediate early baculovirus genes transactivate the p143 gene promoter of *Autographa californica* nuclear polyhedrosis virus. Virology 195, 710–718.
Lu, L., Du, Q., Chejanovsky, N., 2003. Reduced expression of the immediate-early protein IE0 enables efficient replication of *Autographa californica* nucelopolyhedrovirus in poorly permissive *Spodoptera littoralis* cells. J. Virol. 77 (1), 535–545.
Luckow, V.A., Lee, S.C., Barry, G.F., Olins, P.O., 1993. Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. J. Virol. 67 (8), 4566–4579.
Luria, N., Lu, L., Chejanovsky, N., 2012. Conserved structural motifs at the C-terminus of baculovirus protein IE0 are important for its functions in transactivation and supporting hr5-mediated DNA replication. Viruses 4, 761–776.
Lynn, D.E., 1992. A BASIC computer program for analyzing endpoint assays. Biotechniques 12 (6), 880–881.
McCarthy, C.B., Theilmann, D.A., 2008. AcMNPV *ac143* (*odv-e18*) is essential for mediating budded virus production and is the 30th baculovirus core gene. Virology 375, 277–291.
Mikhailov, V.S., 2003. Replication of the baculovirus genome. Mol. Biol. 37 (2), 250–259.
Milks, M.L., Washburn, J.O., Willis, L.G., Volkman, L.E., Theilmann, D.A., 2003. Deletion of *pe38* attentuates AcMNPV genome replication, budded virus production and virulence in *Heliothis virescens*. Virology 310, 224–234.
Nagamine, T., Kawasaki, Y., Matsumoto, S., 2006. Induction of a subnuclear structure by the simultaneous expression of baculovirus proteins IE1, LEF3 and P143 in the presence of *hr*. Virology 352, 400–407.
Neumann, J.R., Morency, C.A., Russian, K.O., 1987. A novel rapid assay for chloramphenicol acetyltransferase gene expression. Biotechniques 5 (5), 444–447.
Nie, Y., 2010. PhD Thesis, Functional comparison and analysis of protein-protein interactions of *Autographa californica* multiple nucleopolyhedrovirus transregulatory proteins of IE0 and IE1. The University of British Columbia, Vancouver, Canada.
Nissen, M.S., Friesen, P.D., 1989. Molecular analysis of the transcriptional regulatory region of an early baculovirus gene. J. Virol. 63 (2), 492–503.
Okano, K., Mikhailov, V.S., Maeda, S., 1999. Colocalization of baculovirus IE-1 and two DNA binding proteins DBP and LEF-3 to viral replication factories. J. Virol. 73 (1), 110–119.
Olson, V.A., Wetter, J.A., Friesen, P.D., 2001. Oligomerization mediated by a helix-loop-helix-like domain of baculovirus IE1 is required for early promoter transactivation. J. Virol. 75 (12), 6042–6051.
Olson, V.A., Wetter, J.A., Friesen, P.D., 2002. Baculovirus transregulator IE1 requires a dimeric nuclear localization element for nuclear import and promoter activation. J. Virol. 76 (18), 9505–9515.
Olson, V.A., Wetter, J.A., Friesen, P.D., 2003. The highly conserved basic domain I of baculovirus IE1 is required for *hr* enhancer DNA binding and *hr*-dependent transactivation. J. Virol. 77 (10), 5668–5677.
Ono, C., Kamagata, T., Taka, H., Sahara, K., Asano, S., Bando, H., 2012. Phenotypic grouping of 141 BmNPVs lacking viral gene sequences. Virus Res. 165 (2), 197–206.
Passarelli, A.L., Miller, L.K., 1993. Three baculovirus genes involved in late and very late gene expression: *ie-1, ie-n* and *lef-2*. J. Virol. 67 (4), 2149–2158.
Pathakamuri, J.A., Theilmann, D.A., 2002. The acidic activation domain of the baculovirus transactivator IE1 contains a virus-specific domain essential for DNA replication. J. Virol. 76 (11), 5598–5604.
Pearson, M., Bjornson, R., Pearson, G., Rohrmann, G.F., 1992. The *Autographa californica* baculovirus genome: evidence for multiple replication origins. Science 257 (5075), 1382–1384.
Pearson, M., Rohrmann, G.F., 1997. Splicing is required for transactivation by the immediate early gene 1 of the *Lymantria dispar* multinucleocapsid nuclear polyhedrosis virus. Virology 235, 153–165.
Pfeifer, T.A., Hegedus, D.D., Grigliatti, T.A., Theilmann, D.A., 1997. Baculovirus immediate-early promoter-mediated expression of the Zeocin™ resistance gene for use as a dominant selectable marker in dipteran and lepidopteran insect cell lines. Gene 188, 183–190.
Rapp, J.C., Wilson, J.A., Miller, L.K., 1998. Nineteen baculovirus open reading frames including LEF-12, support late gene expression. J. Virol. 72 (12), 10197–10206.
Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoints. J. Am. Hyg. 27 (3), 493–497.
Rodems, S.M., Friesen, P.D., 1993. The *hr*5 transcriptional enhancer stimulates early expression from the *Autographa californica* nuclear polyhedrosis virus genome but is not required for virus replication. J. Virol. 67 (10), 5776–5785.
Rodems, S.M., Pullen, S.S., Friesen, P.D., 1997. DNA-dependent transregulation by IE1 of *Autographa californica* nuclear polyhedrosis virus: IE1 domains required for transactivation and DNA binding. J. Gen. Virol. 71 (12), 9270–9277.
Ross, L., Gaurino, L.A., 1997. Cyclohexamide inhibition of delayed early gene expression in baculovirus-infected cells. Virology 232, 105–113.
Slack, J.M., Blissard, G.W., 1997. Identification of two independent transcriptional activation domains in the *Autographa californica* multicapsid nuclear polyhedrosis virus IE1 protein. J. Virol. 71 (12), 9579–9587.
Stewart, T.M., Huijskens, I., Willis, L.G., Theilmann, D.A., 2005. The *Autographa californica* multiple nucleopolyhedrovirus *ie0-ie1* gene complex is essential for wild-type virus replication, but either IE0 or IE1 can support virus growth. J. Virol. 79 (8), 4619–4629.
Taggart, D.J., Mitchell, J.K., Friesen, P.D., 2012. A conserved N-terminal domain mediates required DNA replication activities and phosphorylation of the transcriptional activator IE1 of *Autographa calfornica* multicapsid nucleopolyhedrovirus. J. Virol. 12, 6575–6585.
Theilmann, D.A., Stewart, S.S., 1991. Identification and characterization of the IE-1 gene of *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus. Virology 180, 492–508.
Theilmann, D.A., Willis, L.G., Bosch, B.J., Forsythe, I.J., Li, Q., 2001. The baculovirus transcriptional transactivator *ie0* produces multiple products by internal initiation of translation. Virology 290, 211–223.
Todd, J.W., Passarelli, A.L., Miller, L.K., 1995. Eighteen Baculovirus Genes, Including *lef-11*, *p35*, *39K* and *p47* Support Late Gene Expression. J. Virol. 69 (2), 968–974.
Vanarsdall, A.L., Pearson, M.N., Rohrmann, G.F., 2007. Characterization of baculovirus constructs lacking either the Ac 101, Ac 142 or the Ac 144 open reading frame. Virology 367, 187–195.
Wang, Y., Wu, W., Li, Z., Yuan, M., Feng, G., Yu, Q., Yang, K., Pang, Y., 2007. ac18 is not essential for the propagation of *Autographa californica* multiple nucleopolyhedrovirus. Virology 367 (1), 71–81.
Washburn, J.O., Chan, E.Y., Volkman, L.E., Aumiller, J.J., Jarvis, D.L., 2002. Early synthesis of budded virus envelope fusion protein GP64 enhances *Autographa californica* multicapsid nucleopolyhedrovirus virulence in orally infected *Heliothis virescens*. J. Virol. 77 (1), 280–290.

Washburn, J.O., Trudeau, D., Wong, J.F., Volkman, L.E., 2003. Early pathogenesis of *Autographa californica* multiple nucleopolyhedrovirus and *Helicoverpa zea* single nucleopolyhedrovirus in *Heliothis virescens*: a comparison of the 'M' and 'S' strategies for establishing fatal infection. J. Gen. Virol. 84 (2), 343–351.
Wu, W., Passarelli, A.L., 2012. The *Autographa californica* M nucleopolyhedrovirus ac79 gene encodes an early gene product with structural similarities to UvrC and intron-encoded endonucleases that is required for efficient budded virus production. J. Virol. 86 (10), 5614–5625.
Yamagishi, J., Burnett, E.D., Harwood, S.H., Blissard, G.W., 2007. The AcMNPV *pp31* gene is not essential for productive AcMNPV replication or late gene trascription but appears to increase levels of most viral transcripts. Virology 365, 34–47.