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Baculoviruses deficient in *ie1* gene function abrogate viral gene expression in transduced mammalian cells

Rodica Efrose [1], Luc Swevers [1], Kostas Iatrou *

Insect Molecular Genetics and Biotechnology Group, Institute of Biology, National Centre for Scientific Research "Demokritos", Aghia Paraskevi Attikis, Athens, Greece

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ABSTRACT

One of the newest niches for baculoviruses-based technologies is their use as vectors for mammalian cell transduction and gene therapy applications. However, an outstanding safety issue related to such use is the residual expression of viral genes in infected mammalian cells. Here we show that infectious baculoviruses lacking the major transcriptional regulator, IE1, can be produced in insect host cells stably transformed with IE1 expression constructs lacking targets of homologous recombination that could promote the generation of wt-like revertants. Such *ie1*-deficient baculoviruses are unable to direct viral gene transcription to any appreciable degree and do not replicate in normal insect host cells. Most importantly, the residual viral gene expression, which occurs in mammalian cells infected with wt baculoviruses is reduced 10 to 100 fold in cells infected with *ie1*-deficient baculoviruses. Thus, *ie1*-deficient baculoviruses offer enhanced safety features to baculovirus-based vector systems destined for use in gene therapy applications.

Introduction

Baculoviruses are a class of DNA viruses whose natural host range is restricted to arthropods, mainly lepidopteran insects. Although baculoviruses typically have a very restricted host range, which is usually limited to one or very few closely related species as in the case of *Bombyx mori* nucleopolyhedrovirus (BmNPV; Maeda et al., 1993), the host range of others, e.g., *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), is wider as the virus is capable to replicate in cells from a larger number of lepidopteran species (Kondo and Maeda, 1991). The restriction in the host range of baculovirus species is not determined by their capability to enter and uncoat their DNA in target cells but by their capacity to undergo DNA replication and gene transcription in them. Baculoviruses are also capable of entering mammalian cells through the endocytic pathway from which they escape through pH-dependent fusion of the envelope with the endosome (van Loo et al., 2001). This study also suggested that viral capsids are subsequently transported via actin filaments toward the nucleus into which they enter via the nuclear pores. More recent investigations established that baculovirus entry into mammalian cells occurs by a clathrin-independent mechanism reminiscent of phagocytosis (Laakkonen et al., 2009). Furthermore, it has been shown recently that AcMNPV entry in both Sf9 host cells and mammalian cells can be stimulated by direct fusion with the plasma membrane at low pH (Dong et al., 2010).

Since the discovery that baculoviruses can enter efficiently a wide variety of established mammalian cell lines and primary cells (Hofmann et al., 1995; Boyce and Bucher, 1996), the applications of baculovirus technology include their possible use as gene therapy vectors and vectors for production of vaccine antigens in mammalian systems (Hu, 2006; van Oers, 2006). The advantages of using baculoviruses as mammalian transduction vectors include the ease of vector generation and production in lepidopteran insect cells, the capacity of the vectors to sustain large insertions in their genomes [up to 40 kb; (Cheshenko et al., 2001)], capacity to infect multiple dividing and non-dividing cell types, lack of induction of cytotoxic effects even when used at high multiplicities of infection (moi) (Gao et al., 2002; Kenoutis et al., 2006), and lack of pre-existing immunity in mammalian hosts relative to vectors derived from mammalian viruses. These advantages should, however, be contrasted to some issues related to safety of use, which should be addressed adequately before baculoviruses may be used as gene therapy vectors for humans. Thus, it is well established that baculoviruses may trigger innate immune responses in cultured mammalian cells and humoral ones *in vivo* (Abe et al., 2003; Facciabene et al., 2004; Georgopoulos et al., 2009; Gronowski et al., 1999; Boulaire et al., 2009). Baculoviruses are also readily inactivated by complement *in vivo* (Kaname et al., 2010). Furthermore, endogenous baculovirus genes were recently found to be expressed in mammalian cells transduced by them (Fujita et al., 2006; Kenoutis et al., 2006; Laakkonen et al., 2008), a situation that could both affect the normal physiological state of the cells and contribute to the induction of cellular immune responses against

* Corresponding author. Fax: +30 210 651 1767.
E-mail address: iatrou@bio.demokritos.gr (K. Iatrou).
[1] Authors with equal contribution.

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transduced cells expressing residual viral proteins in recipient hosts subsequent to cell transplantation with *ex vivo* baculovirus-transduced cells.

In baculovirus infections of insect host cells, the protein encoded by the *immediate-early 1* (*ie1*) gene plays a pivotal role in the orchestration of the different phases of the infection cycle (Choi and Guarino, 1995; Kovacs et al., 1991; Pathakamuri and Theilmann, 2002; Prikhod'ko and Miller, 1996). The IE1 protein is a transcription factor required for activation of delayed-early genes as well as repression of the early genes *ie0*, *ie2* and *pe38* (Leisy et al., 1997). In addition, IE1 is essential for DNA replication and late viral gene expression (Pathakamuri and Theilmann, 2002; Slack and Blissard, 1997). Expression of *ie1* and its splice variant, *ie0*, commences very early, at 2 h post-infection (pi). While the levels of IE0 decrease shortly thereafter, the amount of IE1 protein steadily increases in the infected cells and reaches high values at the late phases of infection (Choi and Guarino, 1995; Huijskens et al., 2004). Maintenance of an *ie1* temperature-sensitive mutant virus at the restrictive temperature causes a block in the progression of viral infection (Choi and Guarino, 1995) indicating that IE1 may be considered as the "motor" of the viral infection cycle.

In contrast to the extensive information that exists in relation to transcriptional events in baculovirus-infected lepidopteran cells, little is known about the regulation of baculovirus gene expression during transduction of mammalian cells. Baculovirus gene expression in mammalian cells is considered to be limited to early gene expression, while late genes, which require a viral RNA polymerase for expression, are thought to remain silent (Kenoutis et al., 2006). On the other hand, the activation domain of IE1 is functional in mammalian cells (Dai et al., 2004) and over-expression of IE1 in mammalian cells triggers the activation of baculovirus genes in a manner reminiscent of that induced in insect cells (Liu et al., 2007). This, in turn, suggests that a number of baculovirus gene promoters may be functional in mammalian cells as long as essential viral transcription factors such as IE1 and IE2 are expressed (Liu et al., 2007). In turn, this contention leads to the hypothesis that viral gene transcription in mammalian cells may be abrogated if expression of IE1 (and, preferably, IE2 as well) is inhibited.

In this work, we describe the generation and functional characterization of *ie1* gene-knockout baculoviruses (AcMNPV) produced in transformed Sf21 cell lines over-expressing IE1. After establishing the arrest in viral gene transcription and lack of viral DNA replication in normal insect host cells infected with the *ie1*-knockout virus, we are analyzing the accumulation patterns of viral transcripts in mammalian cells infected with them as compared to cells infected with wild type (wt) virus. Our results establish that mammalian cells infected with *ie1*-knockout viruses accumulate dramatically lower levels of viral transcripts relative to cells infected with wt viruses and suggest that such genetically disabled viruses are safer vehicles for gene therapy applications, especially for cases of transplantation of *ex vivo* transduced cells.

Results

Generation of IE1-expressing Sf21 cells and generation of ie1 knockout viral particles

The generation of an AcMNPV bacmid containing zeocin antibiotic resistance cassette in the place of the *ie1* ORF has been described previously (Stewart et al., 2005). This bacmid, AcBacIE1KO, does not infect Sf9 host cells, thus establishing that the *ie1* gene is essential for viral infection (Stewart et al., 2005). The production of *ie1*-deficient but infectious baculoviruses that could be used for infection of various cell types requires the availability of rescue host cells lines that express constitutively high levels of IE1 protein that could sustain the formation of viral particles from the transfected AcBacIE1KO bacmid genome. However, because of the relatively high copy numbers of *ie1* transgenes in the genomes of the rescue cell lines (generated by transformation with molar ratios of 10 *ie1* expression vectors to 1 puromycin resistance-conferring plasmid) and the importance of the IE1 protein for the initiation and completion of the infectious cycle, considerable selection pressure exists for the deficient virus to acquire the IE1 ORF from the host genome and revert to a fully functional virus.

To avoid homologous recombination between the chromosomally integrated IE1-expressing transgene and the AcBacIE1KO genome, two IE1 expression vectors were constructed, pEA.ie1 and pA.ie1, which expressed the AcIE1 ORF under the control of the silkmoth cytoplasmic actin promoter, in the presence or absence of the BmNPV *hr3* enhancer, respectively (Fig. 1). These expression vectors were completely devoid of sequences located upstream of the AcIE1 ORF but differed at the 3'-ends of their inserts. Specifically, the pEA.ie1 vector contained approximately 0.35 kb of sequences that were also present in the AcBacIE1KO genome [~50 bp of the terminal sequences of the AcIE1 ORF and ~300 bp of adjacent 3'-flanking sequences; (Stewart et al., 2005)], while in the pA.ie1 vector all 3'-flanking sequences of the wt *ie1* gene were removed thus leaving only 50 bp encoding the C-terminus of IE1 as a possible target of recombination with the AcBacIE1KO genome (Stewart et al., 2005).

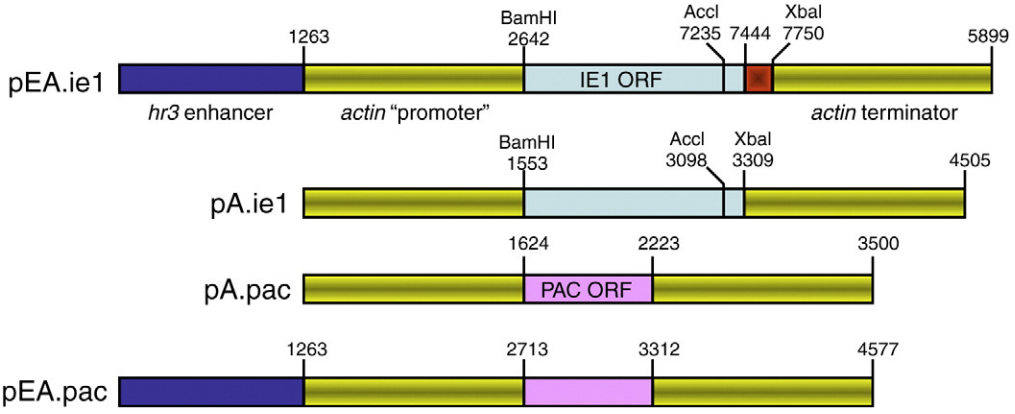

Fig. 1. Schematic overview of IE1 expression constructs pEA.ie1 and pA.ie1 and puromycin selection cassettes pA.pac and pEA.pac. Indicated are the coordinates of the different genetic elements (in nucleotides). Coordinates of ORFs in pA.pac and pEA.pac are indicated from the first nucleotide of the start codon to the last nucleotide of the stop codon. In cassettes pEA.ie1 and pA.ie1, the engineered *Bam*HI restriction site is located 9 nucleotides before the start codon. In pEA.ie1, the position of the stop codon is at 7444. In pEA.ie1, the restriction sites for *Acc*I (at 7235), at the 3'-end of the IE1 ORF, and for *Xba*I (at 7750), at its 3'-flanking region, used for deleting the 3'-flanking region of *ie-1* (see Materials and methods) are indicated. Indicated in red is the 3'-flanking region of *ie-1* that is maintained in pEA.ie1 (from stop codon at 7444 to *Xba*I-site at 7750) but absent in pA.ie1. In pA.ie1, the engineered *Xba*I restriction site (at 3309) is located 2 nucleotides after the stop codon.

Rescue lines were constructed by transforming stably Sf21 cells with the pEA.ie1 or pA.ie1 expression vectors. Both rescue lines expressed robustly functional IE1, as verified by Western blot analysis (Fig. 2A). Moreover, immunofluorescence staining showed that IE1 was present in the nuclei of approximately 50% of the transformed cell populations (Fig. 2B).

When control Sf21 and transformed Sf21/A.ie1 or Sf21/EA.ie1 cells were transfected with AcBacIE1KO DNA, GFP fluorescence was observed only in the transformed cells at 2 days post-transfection, with occlusion body formation becoming evident in 5–10% of the transformed cells at 3–4 days post-transfection [Fig. 2C for results obtained with the Sf21/A.ie1 cell line; identical results were obtained with the Sf21/EA.ie1 cell line (data not shown)]. When supernatants from transfected Sf21/A.ie1 or Sf21/EA.ie1 cells were used to infect new rescue cells, infection became readily evident but again in a similar fraction of the cell population as observed after transfection (Fig. 2D, lower). Thus, although the infection did not spread efficiently, these results nevertheless indicate that the initial transfection had resulted in the production of functional virus capable of undertaking productive infection of rescue cells. In contrast, when the same supernatants were used to infect control Sf21 cells, only very limited GFP fluorescence was observed and no occlusion body formation occurred (Fig. 2D, upper) thus demonstrating that the virus could enter the cells but not infect them productively. Even after eight consecutive passages through the Sf21/A.ie1 or Sf21/EA.ie1 cell lines, productive infection of normal Sf21 cells was not observed, thus demonstrating that the AcBacIE1KO virus was unable to acquire the IE1 ORF from the rescue transgene. Thus, the frequency of homologous or non-homologous recombination events that could lead to the acquisition of a functional *ie1* gene by the nominally *ie1*-knockout viruses in Sf21/A.ie1 and Sf21/EA.ie1 cell lines appears to be minimal making these cell lines suitable for production of AcBacIE1KO baculoviruses, at least for a limited number of passages. However, our observations do not exclude the possibility of generation of *ie-1* revertants in future generations of viral stocks and it is therefore recommended to screen all viral stocks for acquisition of the *ie-1* gene, for instance by PCR or by infection of control host cells.

The titers of the *ie1*-knockout viruses produced in the rescue cell lines that produced IE1 at high levels without providing appreciable targets for homologous recombination between their transgenes and

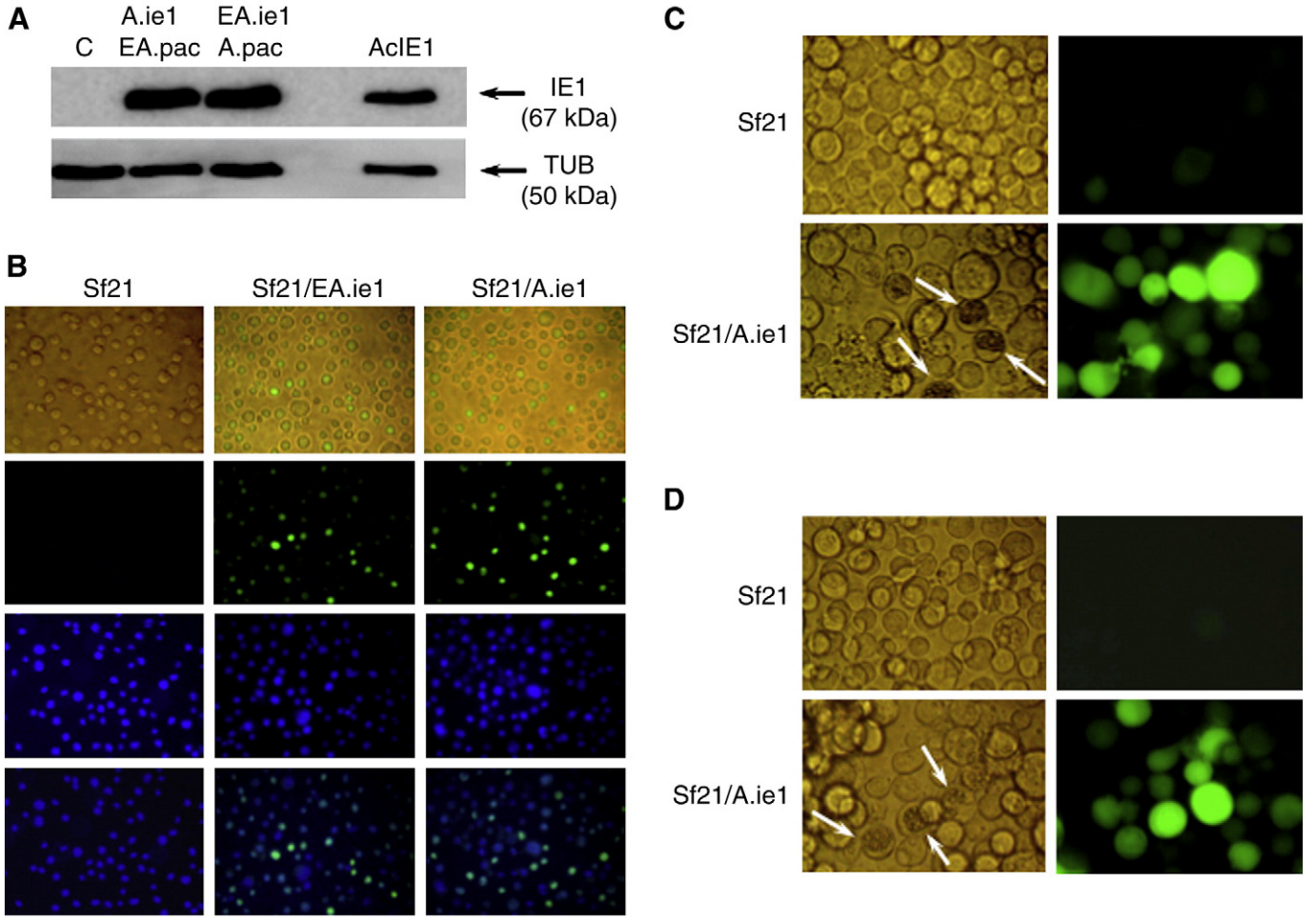

Fig. 2. Evaluation of the rescuing cell lines for IE1 expression and generation of AcBacIE1KO baculovirus. (A) Western analysis of IE1 expression in the two transformed Sf21 cell lines, Sf21/A.ie1 and Sf21/EA.ie1, using a monoclonal antibody raised against AcMNPV IE1 (IE1-4B7). C, uninfected control Sf21 cells; A.ie1/EA.pac, Sf21/A.ie1 cell line; EA.ie1/A.pac, Sf21/EA.ie1 cell line. Each lane was loaded with protein obtained from 250,000 cells. In the lane "AcIE1", a sample was loaded of Sf21 cells infected with AcMNPV. Tubulin staining is included as control. (B) Immunostaining using the same anti-IE1 antibody. Top: bright field and fluorescence. Upper Middle: fluorescence only. Lower Middle: nuclear staining using Hoechst 33238. Bottom: Merge of fluorescence staining of IE1 and nuclear staining by Hoechst 33238. The IE1 antibody stains the nuclei in the cells of the rescuing lines but not those of the control cells. (C) Generation of AcBacIE1KO virus in Sf21/A.ie1 cells. Control Sf21 and transformed Sf21/A.ie1 cells were transfected with 5 μg/ml AcBacIE1KO DNA and analyzed for GFP expression and occlusion body formation at 8 days post-transfection. Left: bright field images. Right: fluorescence images. Arrows point to examples of cells containing occlusion bodies. Note the absence of occlusion bodies from the transfected control Sf21 cells. (D) Propagation of AcBacIE1KO virus. Control Sf21 and transformed Sf21/EA.ie1 cells were infected with supernatants collected from transfected Sf21/A.ie1 cells (shown in C above) and analyzed for GFP expression and occlusion body formation at 8 days pi. Left: bright field images. Right: fluorescence images. Arrows point to examples of cells containing occlusion bodies. Note the limited GFP expression and absence of occlusion bodies in the infected control Sf21 cells.

Table 1
Virus titer determination by quantitative real-time PCR. Stocks of AcBacIE1KO and AcBacWT virus were obtained from Sf21/A.ie1 and wt Sf21 cells, respectively, and consisted of supernatants collected at 10 days post infection.

Baculovirus	Stock	Titer (pfu/ml)
AcBacIE1KO	1	1.83×10^{8}
	2	9.36×10^{5}
	3	8.42×10^{6}
	4	9.36×10^{6}
	5	1.88×10^{6}
AcBacWT	1*	*10^{9} pfu (end-point dilution)*
	2	8.12×10^{7}
	3	7.95×10^{8}

* AcMNPV WT virus titer (10^{9} pfu/ml) was determined by end-point dilution method based on GFP fluorescence and occlusion body formation in Sf21/A.ie1 cells.

the *ie1*-knockout viral genomes were determined by quantitative real-time PCR of viral genome copy numbers in the supernatants of the infected cells and subsequent conversion to plaque forming unit (pfu) per ml equivalents through calibration with a sample containing wt AcMNPV of a known titer. As shown in Table 1, titers of *ie1*-knockout bacmid produced by Sf21/A.Acie1 cells showed considerable variability. Although the concentrations of AcBacIE1KO could reach initially more than 10^{8} pfu/ml equivalents, lower concentrations of approximately 10^{6} pfu/ml equivalents were subsequently obtained from the same cell lines (Table 1). The low quantities of *ie-1* gene knockout virions obtained from the rescue cell line (generally 100- to 1000-fold lower compared to wt virus) are likely due to the limited number of cells that become productively infected, as noted above for the limited number of *ie1*-gene transformed cells that could sustain production of occlusion bodies. Furthermore, while some cells produce occlusion bodies after 4 days, it was also observed that the infection (occlusion body formation) did not spread easily and often remained limited to less than 10% of the cell population (data not shown).

Transcriptional profiling of Sf21 cells infected with ie1-knockout AcMNPV

Normal Sf21 cells were infected with wt and *ie1*-knockout virus at a moi of 5 and the temporal accumulation patterns of viral gene transcripts were assessed by quantitative RT-PCR. For this analysis, several members of each temporal class of genes were examined: *ie1, ie2, pe38* (immediate-early genes); *he65, gp64, dnapol, p143, lef1, lef2, lef3* (delayed-early genes); *p35, vp39, orf141* (late genes); and *p10, polh* (very late genes).

As is evident from the results presented in Fig. 3, only low levels of viral gene expression were observed after infection of the cells with the *ie1*-knockout virus, in agreement with the requirement for *ie1* function at all levels of the baculovirus infection cycle (Choi and Guarino, 1995; Stewart et al., 2005). Interestingly, at the early phases of infection (4 h and 16 h pi), the transcript levels for two viral genes, *ie2* and *pe38*, were higher in cells infected with the *ie1*-knockout than the wt virus (Fig. 3). This likely reflects the function of IE1 as a transcriptional repressor for these genes at the early stages of the infectious cycle (Leisy et al., 1997). At 72 h and 96 h pi, transcript levels could be observed for almost all viral genes in the cells infected with wt virus but for the *ie1*-knockout virus the transcript levels were lower than those of the wt AcMNPV by about 4 to almost 8 orders of magnitude (Fig. 3). Last but equally important, it was also clear that viral DNA replication was aborted in the cells infected with the *ie1*-knockout virus, consistent with the requirement of immediate and delayed early gene expression for viral DNA replication.

Viral gene expression in mammalian cells infected with ie1-knockout virus

We and others have recently reported that baculovirus genes, primarily of the immediate and delayed early classes, are transcribed, albeit at low levels, in mammalian cells (Fujita et al., 2006; Kenoutis

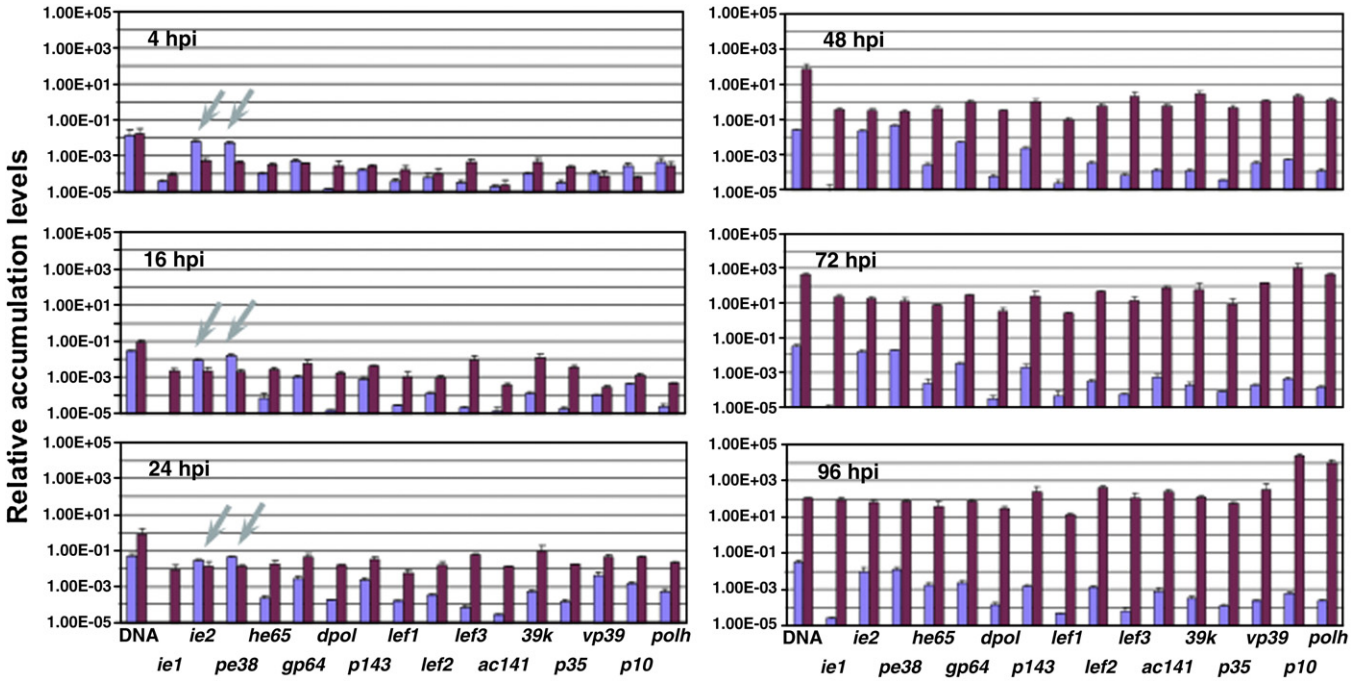

Fig. 3. Temporal accumulation patterns of viral gene transcripts in infected Sf21cells. Sf21 cells were infected with AcBacWT or AcBacIE1KO bacmid virions at a moi of 5 and collected at 4, 16, 24, 48, 72 and 96 h post-infection. Levels of viral transcript accumulation (red bars for AcBacWT, blue bars for AcBacIE1KO) were determined by qRT-PCR using gene-specific primers and relative gene expression levels were calculated with respect to the level of cellular *ß-tubulin* transcripts. For the relative quantification of viral genome copy numbers in insect cells, total DNA isolated from infected Sf21 cells was also subjected to quantitative PCR. Bars show means ± standard error ($N=3$). The arrows refer to the increased expression of *ie2* and *pe38* during early stages of infection with AcBacIE1KO compared to AcBacWT.

et al., 2006) thus putting in doubt the widespread belief that the use of baculoviruses for gene therapy purposes is safe due to the transcriptionally inert nature of baculovirus genomes in the recipient cells (Hu, 2006). To examine whether the deletion of the *ie1* gene from the baculovirus genome also results in a reduction or elimination of residual viral gene expression in mammalian cells, HEK293 cells were infected with AcBacWT or AcBacIE1KO virus. To ensure quantitative infection of the cells, infection was carried out at a nominal moi of 400 for both viruses, which was previously shown to be sufficient for quantitative infection of HEK293 cells (Kenoutis et al., 2006). The results of the qRT-PCR analysis showed that the extent of viral gene transcripts present in cells infected with wt virus were on average 1 to 2 orders of magnitude less abundant than those of the host cell house-keeping *glyceraldehyde 3-phosphate dehydrogenase* (*gapdh*) gene (Fig. 4). Importantly, the same experiments demonstrated that although viral gene transcripts are not eliminated completely from cells infected with the *ie1*-knockout virus, the levels of specific transcript accumulation were one to two orders of magnitude lower (range 11–193 fold) than those found in HEK293 cells infected with AcBacWT (Fig. 4). Interestingly, amongst the least affected viral transcripts were those of the immediate early class (genes *ie2*, *pe38* and *he65* genes whose transcripts were reduced 16, 22 and 17-fold relative to those of the wt virus). In contrast, the transcript levels for most viral genes whose expression is positively regulated by IE1 was found to be much more severely suppressed in the cells infected with the *ie1*-kockout virus (Fig. 4), suggesting that IE1 is capable of acting as a transcriptional activator of baculovirus genes even in a mammalian cell environment.

Discussion

The major transcriptional regulator of baculoviruses, IE1, is an essential factor required at all levels of the infection cycle in host insect cells (Choi and Guarino, 1995). Following transfection of Sf9 host cells with bacmid genomic DNA containing an inactivated *ie1* gene, viral DNA replication and occlusion body formation are not observed (Stewart et al., 2005). Therefore, an important achievement of this work was the generation of infectious baculoviruses that lack the gene encoding the major transcriptional regulator IE1 and the study of their transcriptional competence in infected host insect and mammalian cells. Our results clearly show that *ie1*-knockout viruses not only exhibit a general gene silencing phenotype in insect cells but, also an analogous albeit significantly less pronounced phenotype in mammalian cells, a finding of significance in view of the potential use of baculoviruses as gene therapy vectors.

The production of *ie1*-deficient baculoviruses required the availability of rescue host cell lines that expressed constitutively the IE1 protein at levels that could sustain the formation of viral particles from a transfected bacmid genome in which a bacterial zeocin resistance cassette substituted for the IE1 ORF (Stewart et al., 2005).

Previous studies that attempted to create infectious virions defective for an essential gene, clearly established that such strategy could not succeed when regions of homologous recombination between the transgenes expressed in the rescue cell lines and the viral genomes that were introduced in the same cell lines existed (Kitagawa et al., 2005). In fact, for our case also, we found that generation of wt-like revertants that had acquired an *ie1* gene from the genome of *ie1*-gene transformed cells was possible and dependent on the presence of regions of homology at both the 5' and 3' ends of the *ie1* gene (data not shown).

A somewhat different approach was also employed previously for the generation of AcMNPV deficient in another essential function, GP64 (Kitagawa et al., 2005). Rescuing cell lines, which had incorporated the *gp64* gene from *Orgyia pseudotsugata* multiple nucleopolyhedrovirus (OpMNPV; Monsma et al., 1996), were employed as host cells for the propagation of the deficient virus on the assumption that sequences from OpMNPV were sufficiently heterologous to reduce or eliminate homologous recombination with the AcMNPV genome. However, in that case too it was observed that the cellular rescuing gene could be acquired through a mechanism that apparently involved non-homologous recombination (Kitagawa et al., 2005). Thus, because of the existence of strong selection pressure to acquire an essential gene by the deficient virus, it is important that careful monitoring of viral stocks produced by the rescuing cell lines for the absence of wt-like revertants be carried out by PCR to ensure that only viruses lacking the deleted gene are present in the progeny virus population.

The approach employed in this study established that contrary to the other cases described above, it is possible to generate infectious viruses deficient for an important viral regulator using cell lines that express a rescuing transgene incorporated in the host cell genome, provided that no regions of homologous recombination exist between transgenes and deficient viruses. Using these cell lines, production of *ie-1* knockout virions was achieved although the titers of the obtained viral stocks were significantly lower than those for wt virions (Table 2). The reason for the production of lower amounts of infectious particles is not known at present. A relevant observation is, however, that occlusion body formation occurs only in a limited number of the transformed cells. This does not seem to be caused by lack of expression of IE-1 protein since approximately 50% of the cells in the rescue line express high levels of IE-1 (Fig. 2B). Because infection with wt AcMNPV also occurs at lower efficiency in transformed IE-1-expressing cells relative to non-transformed cells (unpublished observations), it is not unlikely that the continuous expression of *ie-1* in the transformed cells may interfere with baculovirus infectivity or progeny production due to changes in the host cell protein expression profiles. These possibilities are currently under investigation.

Recently, functional ablation of the *ie1* gene during the multiplication of AcMNPV was achieved through RNA interference using gene-specific dsRNA (Schultz et al., 2009). This study confirmed the role of IE1 as an essential factor that regulates both early and late viral gene expression and budded and occluded virus production. Infection of control Sf21 cells with *ie1*-deficient baculovirus confirmed the essential role of the IE1 transactivator (Fig. 3). With the exception of the early expression of the *ie2* and *pe38* genes whose expression is

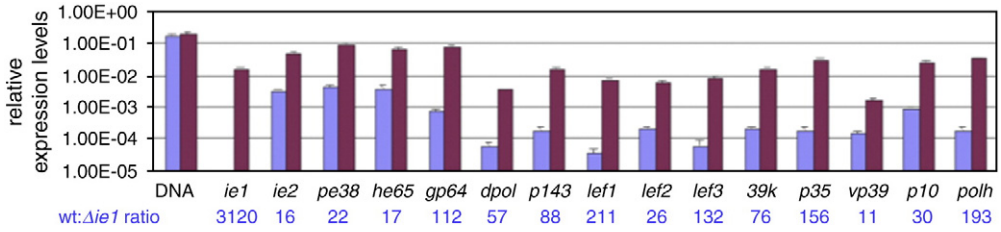

Fig. 4. Accumulation patterns of viral gene transcripts in HEK293 mammalian cells transduced with AcBacWT (red bars) or AcBacIE1KO virus (blue bars) at 3 days post-infection. Viral transcript levels were detected by real-time PCR using gene-specific primers and relative gene expression levels were calculated with respect to the level of cellular *gapdh* transcripts. For the relative quantification of viral genome copy numbers in mammalian cells, total DNA isolated from infected HEK293 cells was also subjected to quantitative PCR. Bars show means ± standard error ($N = 3$).

Table 2
Primers used for real-time qRT-PCR.

Target gene	Forward primer	Reverse primer	RT-PCR product (bp)
Acie1	5'-CAAGATGTGTGCAACGACGAGA-3'	5'-AAACGTGTGGTGCACATCGAC-3'	74
Acie2	5'-CATTGGCGCGAGCTTTTAGA-3'	5'-GCAGCTCGGACAGTTCTTTTTG-3'	93
Acpe38	5'-TGCAGCATCAGGTGAGGACAT-3'	5'-TGTCGTCACGGCCAATTTG-3'	72
Acgp64	5'-CCACCATGGAGAACACCAAGTT-3'	5´-CCTTCAGCCATGGAAGTGATGT-3´	69
Ache65	5'-TTAGAAACGTGCATGCGATCG-3'	5'-CAACCCATTAATTCGCCGTAGA-3'	73
Acdpol	5'-ACAGAAAAAGCGTACCCCTTGC-3'	5'-TGCCCATGTGTTTTATCCAGCT-3'	72
Acp143	5'-CTGAGCAACGACCCTATGCAT-3'	5'-AGCGCATACAGCGGACTATTG-3'	70
Aclef1	5'-ATTGTGTGAGAGAGGCCGTACG-3'	5'-TGAGCGCGTCCAAGTTTGA-3'	65
Aclef2	5'-ATCAAGGCTCTGACGCATTTCT-3'	5'-AAGATGCATGACTTCACCCACA-3'	62
Aclef3	5'-AAAGCGACGAGTTGAACATTGC-3'	5'-CCATTTGCCCAAGTCGGAA-3'	64
Ac141	5'-AGAGATTGAAAGCGCGTACGG-3'	5'-ATTAGCTTCAACGCGTCTCGC-3'	92
Ac39K	5'-AATCACAATGTTGCGCCTCTG-3'	5'-GATGAAATCGCGCGTTAACACT-3'	62
Acp35	5'-CTTTCGAGTACAACCCGATTGG-3'	5´-CGTGTCGTTAATTTCGTGAGCA-3´	64
Acvp39	5'-GACACACGTGAGCTGGACAGAA-3'	5'-GGTATCCCAAAAACAGCGGAA-3'	75
Acp10	5'-CGACATTGTTCCGGATCTTCC-3'	5'-CGAGCGTCTGAATCGAGTTCA-3'	77
Acpolh	5'-GCGGCTGCCCAATAATGAA-3'	5'-CCCAGATGACACGATCGATGA -3'	74
β-tubulin	5'-TTGCATTGGTACACTGGCGA-3'	5'-ACACCAGGTCGTTCATGTTGC-3'	75
gapdh	5'-AGATCATCAGCAATGCCTCCTG-3'	5'-TGGTCATGAGTCCTTCCACGA-3'	95
gfp	5'-GAGCAAAGACCCCAACGAGAA-3'	5'-ACTTGTACAGCTCGTCCATGCC-3'	94

negatively regulated by IE1 (Leisy et al., 1997), the expression of all other baculovirus genes remained at an absolute minimum at all time points investigated (Fig. 3). Most importantly, viral DNA replication was not observed in control Sf21 cells infected with IE1-deficient baculovirus (Fig. 3).

The last but certainly not least important aspect of the functional properties of *ie1*-knockout viruses is related to the safety of their use as gene therapy vectors. One outstanding issue that tends to be ignored by many workers in the field is the residual expression of baculovirus genes in mammalian cells. Previous studies have established the expression of mRNAs encoded by several viral early and delayed-early genes in infected mammalian cells (Fujita et al., 2006; Kenoutis et al., 2006). Furthermore, the expression of at least one viral gene, *ie2*, was sufficiently high to allow detection of the encoded protein by Western blot analysis (Laakkonen et al., 2008). With regard to the impact of viral infection on the physiology of the cells, only minimal differences in host gene expression were previously observed upon baculovirus infection by microarray hybridization analysis (Kenoutis et al., 2006). It should be noted, however, that this analysis was carried out on mammalian cells infected in the absence of trichostatin-A (TSA) treatment, which, in most studies, is used to effect an increase in the efficiency of foreign gene expression in the host cells (Spenger et al., 2004). Moreover, the expression of the β-*actin* gene has been reported to be up-regulated in mammalian cells infected with AcMNPV (Fujita et al., 2006) while a remodelling of the host cell chromatin and an alteration in the size of nuclear structures such as promyelocytic leukaemia nuclear bodies (Laakkonen et al., 2008) appear to also occur in response to infection. Nevertheless, a recent study established that baculovirus infection triggers only mild induction of immune-related factors in mesenchymal stem cells and that transduced cells were well tolerated upon *in vivo* transplantation (Chuang et al., 2009).

Our current study, which compared viral gene expression in mammalian cells infected with wt and *ie1*-deficient baculoviruses, established clearly the functional relevance of IE1 in relation to the overall patterns of viral gene expression in mammalian cells. Although our study concerned only one cell type, it is expected (but remains to be formally demonstrated) that significant reduction in viral gene expression after infection with *ie1*-deficient baculovirus will also be observed in other mammalian cell types that are efficiently transduced by baculovirus, with the possible inclusion of cell types that are involved in the generation of the immune response. Because the levels of viral gene expression in baculovirus-infected mammalian cells treated briefly with TSA can be quite high (for most examined viral genes ranging from ~1% to ~10% of the level of GAPDH mRNA; Fig. 4), the reduction in viral transcription levels effected by the elimination of the *ie1* gene (11- to 211-fold reduction relative to the levels arising from infection with wt baculoviruses; Fig. 4) could reduce the risk of changing the functional properties of the cells and mounting of cellular immune responses against them due to cell surface display of minor quantities of foreign peptides derived from processing of viral proteins, particularly when in the context of a host transplanted with cells transduced *ex vivo* with recombinant baculoviruses. It is therefore believed that the use of *ie1*-deficient baculovirus vectors represents a significant improvement regarding safety issues in the use of baculoviruses as gene therapy vectors.

Materials and methods

IE1 expression and antibiotic resistance plasmids

To clone the AcIE1 ORF under the control of the actin promoter-based expression cassettes (Johnson et al., 1992), the linker fragment

5'-GGCC*GGATCC***CAAC**ATGACGCAAATTAATTTTAA-3'
3'-*CCTAGG***GTTG**TACTGCGTTTAATTAAAATTGCGC-5'

was inserted between an *Eag*I site located 241 bp upstream of the AcIE1 ATG and a *Mlu*I site located in codon #8 of the AcIE1 ORF, thus engineering a *Bam*HI cloning site (italics) and a Kozak initiation sequence (CAAC, bold; Cavener, 1987) immediately upstream of the AcIE1 ATG initiation codon (underlined). The AcIE1 ORF with the engineered translation initiation site together with 307 bp of 3'-flanking sequence was subsequently cloned as a *Bam*HI–*Xba*I fragment into the polylinker of the pEA expression vector (Douris et al., 2006; Lu et al., 1997) to generate expression vector pEA.ie1 (Fig. 1). To eliminate the 307 bp 3'-flanking sequence from the cloned insert, a polymerase chain reaction (PCR) fragment was generated forward primer 5'-CTGGCGAAATTAAAGATGTAGACGTTAG-3' and reverse primer 5'-GCGC*TCTAGA*TTAAAGTTCGAATTTTTTATATTTA-CAATTTAG-3' (underlined inverse complement of terminator codon) and Pfu DNA polymerase (Fermentas). The resultant PCR fragment was subsequently used to replace a 514 bp *Acc*I–*Xba*I fragment in pAcie1 (Guarino and Summers, 1987; Fig. 1) and introduce a unique *Xba*I site (italics in the reverse primer) immediately downstream of the stop codon. The AcIE1 ORF with the engineered translation initiation site and without its 3'-flanking sequence was subsequently cloned as a *Bam*HI–*Xba*I fragment into the pA expression vector (Douris et al., 2006; Lu et al., 1997) to generate expression vector pA.ie1 (Fig. 1). The antibiotic resistance plasmid pBmA.pac that

expresses puromycin acetyltransferase (PAC; Fig. 1) has also been described (Douris et al., 2006). To generate plasmid pEA.pac, an *Eco*RV–*Bam*HI fragment encompassing the PAC ORF was subcloned into the *Sma*I–*Bam*HI-site of the pEA vector (Fig. 1).

Insect cell culture and viruses

IPLB-Sf21AE (Sf21; Vaughn et al., 1977) cultured cells were maintained in IPL-41 medium (Invitrogen) supplemented with 10% FBS at 28 °C (Johnson et al., 1992). Infection of Sf21 cells with AcMNPV, amplification of viral stocks and determination of 50% tissue culture infectious doses were carried out as described (O'Reilly et al., 1992).

Stable transformation of Sf21 cells

Sf21 cells were transfected with different versions of *ie1* gene expression vectors and the puromycin resistance-conferring plasmids pA.pac or pEA.pac at molar ratios of 10:1 using lipofectin (Invitrogen) as transfection agent according to established protocols (Johnson et al., 1992). IE1-expressing cell lines were selected following antibiotic selection (15 μg/ml of puromycin; Applichem) and expanded as described previously (Farrell et al., 2000).

Generation of ie1 gene knockout AcMNPV

DNA was prepared from bacteria containing the AcMNPV *ie1* gene knockout bacmid DNA (AcBacIE1KO; Stewart et al., 2005) and its wt counterpart (AcBacWT) using the Qiagen Large construct kit and used at 2–5 μg/ml to transfect untransformed Sf21 cells or IE1-expressing rescue Sf21 lines using lipofectin (Johnson et al., 1992). Both the AcBacIE1KO and AcBacWT viruses were expressing the ORF of the green fluorescence protein (GFP) protein under the control of the viral *p10* gene promoter and were also polyhedrin-positive because of the insertion of a complete copy of the AcMNPV *polyhedrin* gene by Tn7-mediated transposition using appropriate pFastBac vectors (Stewart et al., 2005). The progression of viral infection was followed by GFP expression and occlusion body formation in the infected cells.

Western blot analysis

Equivalent aliquots from normal and IE1-expressing Sf21 cells (Sf21/EA.ie1, Sf21/A.ie1) were harvested and total extracts were prepared by lysis in cracking buffer (Koelle et al., 1991) for Western blot analysis. Protein samples were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) and transferred to nitrocellulose membrane (Hybond-ECL, Amersham) using a Bio-Rad Mini-Protean II liquid transfer apparatus according to the manufacturer's protocols. Immuno-detection was performed with standard protocols using an anti-AcMNPV IE1 mouse monoclonal antibody (IE1-4B7; generously provided by Dr. Linda Guarino, Texas A&M University) at a 1:1000 dilution (Stewart et al., 2005). After incubation with 1:10000 dilution of goat anti-mouse IgG-horseradish peroxidase-conjugated antibody, signals were detected using the enhanced chemiluminescence system (ECL, Amersham). Anti-tubulin antibodies (AbD Serotec) were used at 1:1000 in conjunction with ant-rat-HRP (Chemicon) at 1:3000.

Immunofluorescence

Analysis of protein localization by immunofluorescence microscopy was carried out according to Labropoulou et al. (2008). Two hundred thousand wt or transformed Sf21 cells were plated onto poly-L-lysine coated micro-slides in a volume of 200 μl and allowed to attach for 2 h at room temperature. Subsequently, cells were washed twice for 5 min with phosphate-buffered saline (PBS), fixed with 4% (w/v) paraformaldehyde in PBS for 10 min at room temperature, washed with PBS three times, permeabilized with 0.1% (v/v) Triton X-100 (in PBS) for 5 min at room temperature, washed three times with PBS for 5 min and then incubated in 3% (w/v) BSA in PBS for 1 h at room temperature. After blocking, the cells were incubated with the AcMNPV IE1 monoclonal antibody (1:500 dilution in 1% BSA in PBS) overnight at 4 °C in a humidified chamber, washed three times with PBS, incubated with a secondary antibody (FITC anti-mouse, 1:500 dilution in PBS containing 1% BSA) for 2 h at room temperature, in a humidified chamber protected from light and washed three times with PBS. For nuclear staining, cells were incubated with Hoescht (0.5 μg/ml) for 5 min at room temperature. Finally, cells were examined for appearance of fluorescence under an Axiovert 25 CFL epifluorescence inverted microscope (Zeiss, Germany).

Baculovirus-mediated transduction of mammalian cells

Human embryonic kidney 293 cells (HEK293; American Tissue Culture Collection) were infected for 8 h with wt or *ie1*-knockout AcMNPV bacmids in Dulbecco's PBS at a multiplicity of infection (moi) of 400 at 28 °C as described (Kenoutis et al., 2006). Trichostatin A (TSA) (Applichem) at 1 μM was added to the cells at the end of the infection period for 24 h and the cells were collected and analyzed for the presence of viral transcripts at 72 h post-infection (pi).

Determination of baculovirus genome and gene transcript copy numbers in infected cells

Aliquots of infected Sf21 and HEK293 cells were harvested at various times pi and subjected to total RNA isolation using Trizol® reagent (Invitrogen). To eliminate traces of genomic DNA contamination, total RNA samples were treated with 0.1 U of RQ1 RNase-free DNase I (Promega, Madison, WI) per μg of RNA at 37 °C for 30 min. First-strand cDNA was synthesized from 2 μg of DNase-treated total RNA using oligo(dT) as primer and SuperScript II reverse transcriptase (Invitrogen). Quantitative (real time) RT-PCR (qRT-PCR) reactions were carried out using the Mx3000P QPCR System (Stratagene) with Maxima ™ SYBR Green qPCR Master mix (Fermentas), gene-specific primers at a final concentration of 0.5 μM each and cDNA template equivalents corresponding to 100 ng aliquots of reverse-transcribed RNAs. PCR cycling started with initial activation of Taq polymerase (HyTest Ltd) at 95 °C for 10 min, followed by 40 cycles of 95 °C for 20 s and 60 °C for 1 min. The quality of qRT-PCR reactions was monitored by dissociation curve analysis and agarose gel electrophoresis on a 3% (w/v) gel. To normalize small differences in cDNA template inputs, the expression levels of the *ß-tubulin* gene of *Spodoptera frugiperda* (Genbank accession number AF548017) and the human *gapdh* gene in HEK293 cells were used as internal reference controls. The relative expression levels of the target genes (X) were calculated as ratios to the reference gene transcripts (R), as $(1+\mathrm{E})^{-\Delta Ct}$, where differences in threshold cycles (ΔC_t) were calculated as ($C_t^X - C_t^R$). PCR efficiency (E) for each amplicon was calculated using the LinRegPCR software (Ramakers et al., 2003). All qRT-PCR reactions were performed as triplicates for the infection of both insect and HEK293 cells. Forward and reverse primers to detect specific transcripts (Table 2) were designed using Primer Express 1.5 software (Applied Biosystems, Darmstadt, DE). For the relative quantification of viral genome copy numbers in insect and mammalian cells, total DNA was isolated from infected Sf21 and HEK293 cells (Iatrou and Swevers, 2005). qPCR reactions were employed using as template 40 ng of genomic DNA, *ie2* gene-specific primers at a concentration of 0.5 μM and Maxima ™ SYBR Green qPCR Master mix (Fermentas.) The analysis of the qPCR reactions was carried out in a similar way as for the relative gene expression level determinations using as standards the same reference genes.

Virus titer determinations by qRT-PCR

All baculoviruses stocks were diluted 20-fold and the titer or titer equivalent of each dilution, for wt and *ie1* gene knockout virus, respectively, was determined by qPCR analysis. For the qPCR reactions, 2 μl of each diluted virus stock was used as template in a final volume of 15 μl which contained Maxima ™ SYBR Green qPCR Master mix (Fermentas), *gp64* gene specific primers (Table 2) at a final concentration of 0.5 μM and 20 ng of Sf21 genomic DNA as internal control for amplification of the *Spodoptera* β-*tubulin* gene sequences. The qPCR assays were performed as described above and monitored by melting curve analysis and agarose gel electrophoresis on a 3% (w/v) gel. Unknown virus titers were calculated by a modification of a previously described method (Lo and Chao, 2004) that employed a comparative quantification using the MxPro™ QPCR Software (Stratagene). The relative quantities of the unknown samples as well as the calibrator sample were calculated relative to the β-*tubulin* internal control. Gene copy numbers determined by qRT-PCR analysis were converted to plaque forming units per ml (pfu/ml) by comparison with the gene copy numbers of the calibrator sample, i.e., a sample of wt AcMNPV whose titer was determined based on GFP fluorescence and occlusion body formation in end-point dilution assays. It should be noted that for the case of the *ie1*-deficient virus, the term pfu/ml actually reflects numbers of viral particles per ml.

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