Journal of General Virology (2007), **88**, 2176–2184 **DOI** 10.1099/vir.0.82664-0

Stimulation of baculovirus transcriptome expression in mammalian cells by baculoviral transcriptional activators

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Received 24 October 2006
Accepted 24 April 2007

Autographa californica multiple nucleopolyhedrovirus (AcMNPV), the type species of the family *Baculoviridae*, is an insect-specific virus that can enter a variety of mammalian cells. The potential of this versatile virus for protein expression or gene therapy in mammalian cells has become the focus of many studies. In most mammalian cells, transduced AcMNPV genes are either not expressed or expressed at an extremely low level. Here, we studied the effects of the two major AcMNPV *trans*-activators, IE1 and IE2, on the activation of AcMNPV genome in Vero E6 cells. Microarray analysis showed that when IE1 was overexpressed, it significantly activated genes *gp64* and *pe38*, and upregulated *ie2*, *he65*, *pcna*, *orf16*, *orf17* and *orf25*. Although, there were only two genes, *pe38* and *orf17*, that were activated by IE2, we discovered interestingly that the combination of IE1 and IE2 factors had a synergistic effect on activation of the AcMNPV genome in mammalian cells, and activated around 38 %, or 59 out of the 155 genes placed on the microarray. This is the first detailed study of baculoviral transcription regulation in mammalian cells, and it shows that the baculoviral genome can be activated in a mammalian system, and also that the two major *trans*-activators, IE1 and IE2, play a central role in this activation.

INTRODUCTION

Baculovirus expression systems have become one of the most versatile eukaryotic systems for protein expression (Kost *et al.*, 2005). *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV), the most characterized member of the family *Baculoviridae*, is a model virus routinely used for foreign protein expression in insect cells. In recent years, there has been increasing interest in its use as a vehicle for delivering foreign genes into mammalian cells for gene expression or gene therapy (Boyce & Bucher, 1996; Hofmann *et al.*, 1995; Hu *et al.*, 2003; Huser & Hofmann, 2003; Huser *et al.*, 2001; Shoji *et al.*, 1997), or as an antigen-presenting, immune-stimulating particle (Abe *et al.*, 2005, 2003; Chang *et al.*, 2004). These wide-ranging potential applications of baculovirus in mammalian systems provide an incentive for an in-depth study of its gene regulation pathways in mammalian cells.

The 133 kb double-stranded, circular DNA genome of AcMNPV has been completely sequenced (Ayres *et al.*, 1994) and is predicted to contain 155 open reading frames (ORFs). In insect cells, these genes are expressed in three temporally ordered phases: early, late and very late. The early genes are further divided into two subclasses: the immediate-early (*ie*) type and the delayed-early type genes. The *ie* genes require no viral factor for activation and are believed to govern the instigation of the baculovirus life cycle within host cells. Two of these genes are *ie1* and *ie2*, the major *trans*-activators of baculovirus. Both of them are involved in the activation of late expression factor genes, which are necessary for virus propagation (Lu & Miller, 1995).

Extensive studies of genes *ie1* and *ie2* and their protein products (IE1 and IE2) have been carried out in insect cells. Mutational and deletion studies of *ie1* and *ie2* genes have previously been used to study their domain functions, and their effects on viral viability and infectivity (Kovacs *et al.*, 1992; Prikhod'ko *et al.*, 1999; Yoo & Guarino, 1994). The IE1 protein *trans*-activates several viral promoters, including promoters of the genes *39K* (Guarino & Summers, 1986), *p35* (Nissen & Friesen, 1989), *gp64* (Blissard & Rohrmann, 1991), *p143* (Lu & Carstens, 1993) and *he65* (Murges *et al.*, 1997). Some of these promoters are greatly enhanced by being *cis*-linked to palindromic sequences from AcMNPV homologous repeat (*hr*) regions (Leisy *et al.*, 1995), which contain IE1-binding sites (Choi & Guarino, 1995). The IE2 protein has been shown to *trans*-activate viral promoters of *ie1*, *ie0* and *ie2* genes (Carson

Supplementary tables are available with the online version of this paper.

et al., 1991; Prikhod'ko *et al.*, 1999). It also augments IE1 activation of the promoter of *39K* (Carson *et al.*, 1988).

In transient expression studies, IE1 protein was shown to activate baculoviral early promoter of *he65* in BHK-21 cells (Murges *et al.*, 1997). In another study, the acidic activation domain of AcMNPV IE1 protein was shown to be active in both NIH-3T3 and CHO cells (Dai *et al.*, 2004). When whole baculoviruses were transduced into mammalian cells, *ie1* gene transcript was detected in AcMNPV-transduced HeLa14 cells on an AcMNPV DNA microarray (Fujita *et al.*, 2006), and in *Bombyx mori* NPV-transduced BEK293 cells and rat primary Schwann cells by RT-PCR (Kenoutis *et al.*, 2006). The presence and functionality of IE1 in these mammalian cells convinced us to explore transcriptional pathways of baculovirus genes in mammalian cells.

In this study, we designed a novel approach to examine the activation of the AcMNPV transcriptome by IE1 and IE2 in a mammalian system. Firstly, we discovered that Vero E6 cells are ideal for this type of study because of their high sensitivity to baculovirus transduction and extremely low levels of endogenous baculoviral gene expression. Secondly, proper *ie* gene expression was achieved in Vero E6 cells by using the cytomegalovirus immediate-early (CMVie) promoter to drive the baculoviral genes, which were delivered into the cells by recombinant baculoviruses. Lastly, the IE1- or IE2-activated AcMNPV transcriptome within the transduced Vero E6 cells was analysed using an AcMNPV DNA microarray. Using this method, we have identified novel sets of baculoviral genes activated by IE1 or IE2 in a mammalian system. More importantly, we also discovered that these two *ie trans*-activators had a synergistic effect on AcMNPV genome activation. This is the first study to draw a clear correlation between baculoviral *trans*-activators and AcMNPV genome activation in a mammalian system.

METHODS

Construction of expression plasmids. For the IE1 functional study in Vero E6 cells, the *ie1* gene was placed under the control of the CMVie promoter to enable its expression in mammalian cells. The coding sequence of *ie1* was PCR amplified from total AcMNPV genomic DNA using the PCR primers IE1 F and IE1 R (Supplementary Table S1 available in JGV Online). The gene fragment was inserted into a modified pTriEx-3 (Novagen) transfer vector in which the p10 and T7 promoters had been deleted, leaving the inserted foreign gene to be driven solely by the CMVie promoter (pTriEx-del). The resulting plasmid was designated pAcIE1. Other plasmids used for the IE1 functional study included the previously constructed pAcE (CMVie promoter-driven *egfp*), pA^{h}cmE (*hr*-CMVm promoter-driven *egfp*) and pApE (polyhedrin promoter-driven *egfp*) (Gossen & Bujard, 1992; Lo *et al.*, 2002) (Fig. 1a).

A reporter cassette containing a *dsR2*-coding region driven by both SV40 (from pcDNA3; Invitrogen) and CMVm (Lo *et al.*, 2002) promoters was inserted into the *Xho*I site of the modified TriEx plasmid to make pAscmR (Fig. 2a). Baculoviruses containing this reporter cassette were able to express DsR2 protein in both

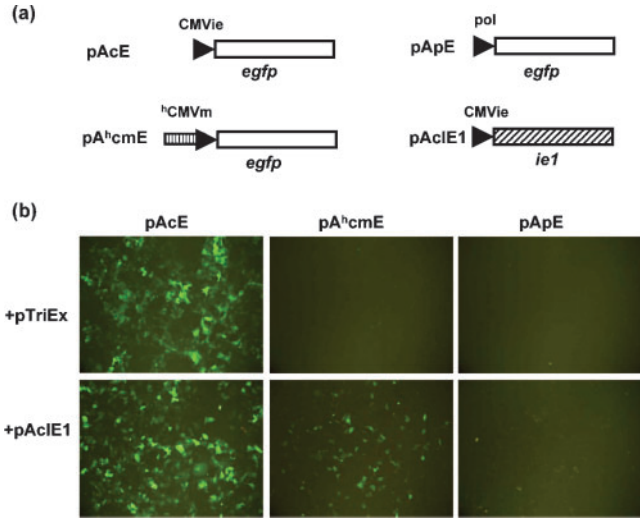

Fig. 1. IE1 functions as a *trans*-activator in Vero E6 cells. (a) Schematic diagram of pAcIE1 (plasmid expresses IE1) and reporter plasmids pAcE, pA^{h}cmE and pApE. (b) The three reporter plasmids were co-transfected with pTriEx (vector only) or pAcIE1. The mammalian CMVie promoter of pAcE acted as the positive control. The promoter of *polyhedrin* (pol) expressed by plasmid pApE served as the negative control. The *hr*-CMVm promoter (from plasmid pA^{h}cmE) was shown to be active only in the presence of IE1.

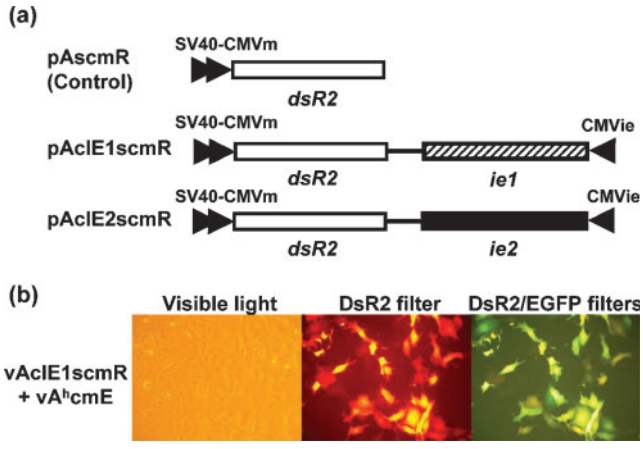

Fig. 2. Construction of IE1 and IE2 expression vectors, and functional test for vAcIE1scmR. (a) Schematic diagram of IE1/IE2 expression vectors for generating recombinant baculoviruses. All expression cassettes were cloned between *Nco*I and *Xho*I sites on pTriEx-del. Recombinant baculoviruses vAscmR, vAcIE1scmR and vAcIE2scmR were made from these expression vectors. (b) Co-transduction of Vero E6 with vAcIE1scmR and vA^{h}cmE, each at an m.o.i. of 10. Photos were taken 48 h post-transduction. Transduction of vAcIE1scmR and vA^{h}cmE viruses induced red and green fluorescence, respectively. Only cells expressing IE1 were able to induce EGFP production from the vA^{h}cmE virus.

mammalian (SV40) and insect cells (CMVm); thus, this simplified the process of single clone purification in insect cells and it doubled as a visual marker for transduction in mammalian cells. Both *ie1* and *ie2* gene fragments were amplified as above and placed under the CMVie promoter of pAscmR, to make pAcIE1scmR and pAcIE2scmR (Fig. 2a). All plasmid constructions were confirmed by DNA sequencing. PCR primers for generating the reporter cassette are also listed in Supplementary Table S1 (available in JGV Online).

Recombinant baculoviruses production in insect cells. The *Spodoptera frugiperda* IPLB-Sf21 (Sf21) cell line was cultured in 10 % fetal bovine serum (FBS)-supplemented TC100 insect medium (Sigma) at 26 °C. Baculoviruses were prepared according to the standard protocols described by O'Reilly *et al.* (1994). Briefly, the expression vectors shown in Fig. 2(a) were co-transfected with vAcRP23.Laz (PharMingen), a linearized viral DNA of AcMNPV, into Sf21 cells using Cellfectin (Life Technologies). The resulting baculoviruses were termed vAscmR, vAcIE1scmR and vAcIE2scmR. Homologous recombination occurs at the site of the *polyhedrin* gene, and produces a baculovirus lacking this gene. Recombinant baculoviruses were isolated through end-point dilutions using DsR2 as the marker, and their titres were determined by quantitative PCR (Lo & Chao, 2004).

Mammalian cell culture and viral transduction conditions. African green monkey kidney epithelial Vero E6 cells were cultured in the presence of 10 % FBS, 100 U penicillin ml^{-1} and 100 μg streptomycin ml^{-1} in minimum essential medium (MEM; Gibco) at 37 °C and 5 % CO_2. Baculoviruses were transduced into Vero E6 cells at an m.o.i. equal to 10. Transduced cells were harvested at various time points by trypsin digestion, and total RNA was extracted using the Qiagen RNeasy mini kit. The quantity and quality of the RNA were checked by a NanoDrop ND-1000 Spectrophotometer (J & H Technology).

Construction of AcMNPV cDNA microarray. All of the 154 ORFs of AcMNPV were amplified by PCR, purified using the QIAquick PCR purification kit (Qiagen) and spotted in duplicate onto GAPS-2 coated slides (Corning). Most of the PCR amplified products were 300–500 bp in length. A set of 10 artificial cDNA fragments from the Stratagene SpotReport Alien cDNA Validation System were also spotted onto glass slides as controls (Stratagene), together with the corresponding exogenous RNA spiked into the labelling reaction; these control spots were used to normalize both dye incorporation efficiencies and signal intensities between different arrays. After spotting, the glass slides were UV-cross-linked at 300 mJ cm^{-2} and blocked for 20 min in 1.55 % succinic anhydride and 84.5 mM sodium borate in N-methyl-2-pyrrolidone.

Hybridization of AcMNPV transcriptome. Recombinant baculoviruses vAscmR (reporter-only control), vAcIE1scmR and vAcIE2scmR were transduced into Vero E6 cells at an m.o.i. of 10. After 48 h, total RNAs from these transduced cells were examined by the AcMNPV cDNA microarray. Total RNA (20 μg) from these cells was reverse-transcribed using SuperScript II (Invitrogen). Probe synthesis, purification and microarray washing were conducted as described previously (Zheng *et al.*, 2004). We used two-colour microarrays, where the total cDNA from vAscmR and vAcIE1scmR (or vAcIE2scmR) transductions were labelled with cy3 and cy5 dyes, respectively. The cy5- and cy3-labelled cDNAs were mixed and applied onto a single AcMNPV microarray block. The microarray was hybridized with a MAUI hybridization system (BioMicro Systems), and then examined using an Axon 4000B scanner (AronInstrument). The levels of total AcMNPV transcripts from both cy5- and cy3-labelled samples were analysed by Axon GenePix Pro 6.0 software. The numerical data obtained were normalized by comparison to the signal intensities of the spike cDNA controls (Stratagene).

Construction of luciferase plasmids. Reporter plasmids (pxL where x is a baculoviral promoter) were constructed using the promoter-less pGL3-basic luciferase vector (Promega) as the backbone. Promoter region of genes: *ie1*, *ie2*, *pe38*, *gp64*, *39K*, *he65*, *orf16*, *orf17*, *orf25* and *pcna* (up to 500 bp) were amplified by PCR and cloned into the pGL3 vector. Primer sequences used to amplify each promoter are given in Supplementary Table S1 (available in JGV Online).

Transfection of Vero E6 cells. Cells (1×10^4) were seeded in each well of a 96-well plate, and the plate was incubated with 5 % CO_2 at 37 °C 24 h prior to the experiment. Transfection was performed using Lipofectamin 2000 as the transfection reagent, following the protocol from the manufacturer (Invitrogen). For each well, 30 ng reporter plasmid (pxL) DNA was mixed with 70 ng effector plasmids (pAcscmR, pAcIE1scmR or pAcIE2scmR) in 10 μl serum-free MEM. A volume of 0.3 μl Lipofectamin 2000 was diluted in 20 μl serum-free MEM and then incubated at room temperature for 5 min, before being combined with the diluted DNA and incubated at room temperature for 20 min. A volume of 30 μl serum-free MEM was then added to each sample before the samples were added to the Vero E6 cells. Cells were incubated at 37 °C with 5 % CO_2 for 4 h before changing the medium to 10 % FBS/2 % antibiotic MEM. Cells were incubated further at 37 °C with 5 % CO_2 for another 48 h before their luciferase activity was measured.

Luciferase assay. After the serum-containing medium was removed, transfected cells in a 96-well plate were washed with Dulbecco's PBS (Invitrogen), then lysed with 100 μl culture cell lysis reagent [100 mM potassium phosphate (pH 7.8), 1 mM EDTA, 10 % glycerol, 1 % Triton X-100 and 2 mM β-mercaptoethanol]. The plate was placed on a shaker at 200 r.p.m. for 20 min at 4 °C to complete cell lysis. Cell lysates were transferred into a 96-well conical-based Nunc MicroWell plate and centrifuged at 1885 ***g*** for 30 min at 4 °C. After centrifugation, 20 μl supernatant from each well was combined with 180 μl luciferase activity reagent [25 mM Tricine (pH 7.8), 15 mM potassium phosphate (pH 7.8), 15 mM $MgSO_4$, 4 mM EGTA, 1 mM ATP and 0.1 mM dithiothreitol] in a luciferase assay plate. Luciferase activity was measured on a luminometer (Lumat LB 9501; Berthold) by injecting 50 μl 0.2 mM luciferin (Promega) into each well. The protein concentration of each well was measured using a Coomassie protein assay kit (Pierce).

RESULTS

IE1 is functional in Vero E6 cells

There is no previous report of IE1 functionality in Vero E6 cells. For a functional assay, we co-transfected each of the three reporter plasmids, pAcE, $pA^{h}cmE$ and pApE, into Vero E6 cells with either pTriEx-3 (vector only) or pAcIE1 (Fig. 1). Unlike the full-length CMVie promoter with enhancer element, the CMV minimal (CMVm) promoter, containing the sequence between −53 and +75 region of CMVie, is not active in mammalian cells (Gossen & Bujard, 1992) and requires the *hr* viral fragment for effective activation in Sf21 insect cells (Lo *et al.*, 2002). We hypothesized that this activation is IE1-dependent, as *hr* contains IE1-binding motifs (Leisy *et al.*, 1995). The pAcE-positive control expressed enhanced green fluorescent protein (EGFP) with or without IE1 protein. The pApE-negative control showed that the promoter of *polyhedrin* was not active in mammalian cells as reported previously (Fujita

et al., 2006; Kenoutis *et al.*, 2006). As predicted, the *hr*-CMVm promoter was clearly dependent on IE1 for *trans*-activation (Fig. 1b). This result was further confirmed when the two recombinant baculoviruses vA^{h}cmE (made from plasmid pA^{h}cmE) and vAcIE1scmR were co-transduced into Vero E6 cells (Fig. 2b). The former virus expresses EGFP by the *hr*-CMVm promoter; and the latter virus expresses both IE1 and DsR2 proteins by CMVie and SV40 promoters, respectively. Using an m.o.i. of 10 for each virus, a large proportion of cells were transduced by both viruses. Fig. 2(b) clearly shows that the green fluorescence from the *egfp* gene of vA^{h}cmE was only present in cells also emitting red fluorescence (from expression of the *dsR2* gene from vAcIE1scmR). This not only reconfirmed that *hr*-CMVm promoter requires IE1 for activation, but also established that baculovirus vAcIE1scmR can effectively express functional IE1 protein in Vero E6 cells. As the transduced virus contains a complete set of AcMNPV genes (apart from the *polyhedrin* gene), we hypothesized that upon the expression of IE1 there will be transcripts of IE1-responsive genes from AcMNPV genome within the transduced mammalian cells.

Hybridization of baculovirus transcripts onto an AcMNPV microarray

To observe the activation of AcMNPV transcriptome by IE1 and another early *trans*-activator, IE2, within Vero E6 cells, we hybridized total cellular cDNAs from vAcIE1scmR- and vAcIE2scmR (cDNAs labelled with cy5)-transduced Vero E6 cells onto an in-house AcMNPV DNA microarray, together with a wild-type baculovirus control, vAscmR (cDNAs labelled with cy3). No significant level of green cy3-labelled AcMNPV transcript was seen; the only spots were from labelled positive control DNA (Fig. 3a). In contrast, within vAcIE1scmR- or vAcIE2scmR-transduced Vero E6 cells, several viral genes were clearly activated because of IE1 or IE2 *trans*-activation (Fig. 3b, c). The activation effects of IE1 and IE2 were synergistically enhanced when both vAcIE1scmR and vAcIE2scmR viruses were co-transduced into the same pool of Vero E6 cells (Fig. 3d). This result indicates that some AcMNPV genes require the cooperative effects of both IE1 and IE2 *trans*-activators for transcription to commence.

Identification of baculoviral genes activated by IE1

In order to discover the extent of IE1 activation, we analysed the microarrays hybridized with vAcIE1scmR-transduced Vero E6 cell cDNA for the presence of baculoviral transcripts (Fig. 3b). On the AcMNPV microarray, the highest value for negative control spots was 127 ± 16 fluorescence units (F.U.); therefore, we chose a cy3 or cy5 with an F.U. of 180 as a lowered cut-off point for positive results. At 48 h post-transduction, we found that AcMNPV genes *gp64*, *pe38*, *ie2*, *he65*, *orf16* and *orf17* were significantly transcribed in the vAcIE1scmR-transduced sample, but no transcription of these genes was observed in the vAscmR control (Fig. 3a). One surprising finding was that the level of early and late viral surface glycoprotein *gp64* gene expression was extremely high, surpassing the expression level of the *ie1* gene, which was driven by mammalian CMVie promoter.

To obtain the onset time and expression profile of these genes that were activated by IE1, we performed time-course studies of IE1-activated AcMNPV transcriptomes in Vero E6 cells. The total RNA from vAcIE1scmR-transduced cells was harvested at 4, 12, 24, 48, 72 and 96 h post-transduction. These samples were analysed with AcMNPV microarrays, and profiles of genes activated by IE1, including *pe38*, *ie2*, *he65*, *orf16*, *orf17*, *orf25* and *pcna*, are shown in Fig. 4(a). The surprisingly strong strength of the promoter of *gp64* in Vero E6 cells was compared with the CMVie-driven *ie1* gene as shown in Fig. 4(b). The levels of

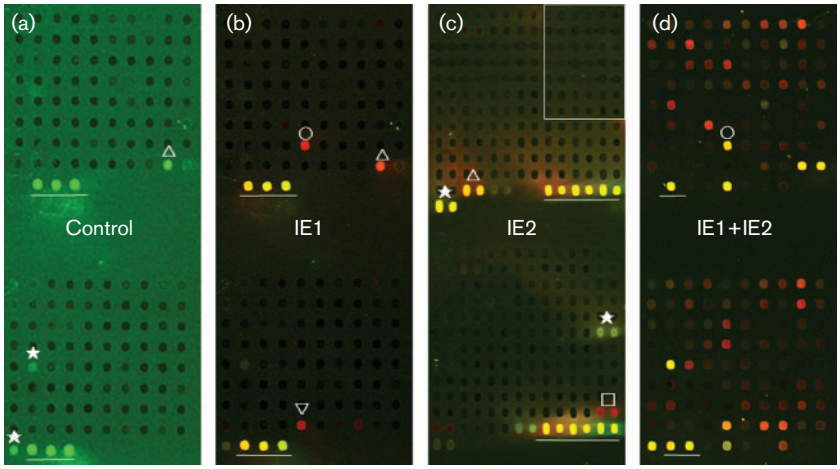

Fig. 3. Hybridization of AcMNPV microarrays. Baculovirus microarrays hybridized with cDNA from Vero E6 cells transduced with control vAscmR (a) and recombinant baculoviruses expressing IE1 (b), IE2 (c) or IE1+IE2 (d). In each experiment, the vAscmR-virus (expressing *dsR2*)-transduced Vero E6 cDNA was labelled with cy3 as the control. The cy5-labelled cDNA samples showed up red on the panels representing activated AcMNPV genes. Except for control cDNAs, no cy3-labelled green spot can be seen in (a), indicating that no AcMNPV gene transcript was detected in the vAscmR-transduced samples. The top-right corner of the IE2 array (c) is from a sister block on the same microchip. ○, gp64; ▽, IE1; □, IE2; ☆, actin; △, *dsR2*; the spike-in controls are underlined.

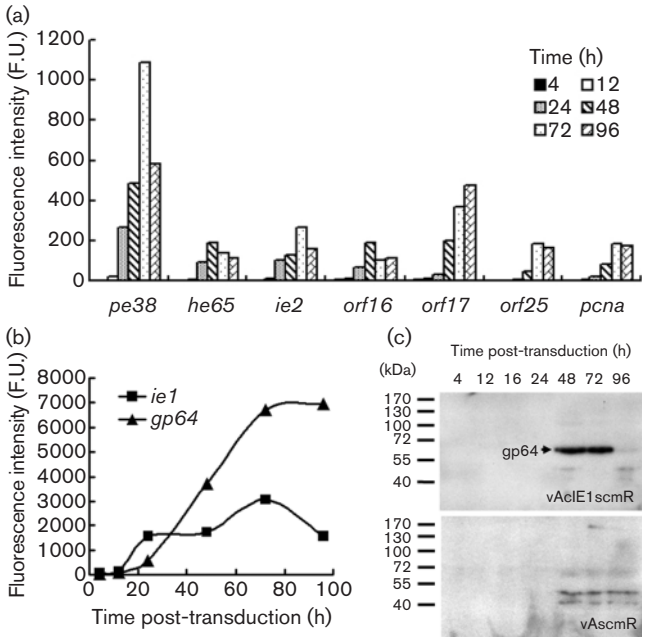

Fig. 4. Activation of AcMNPV transcripts by IE1. Fluorescence measurements were taken from five AcMNPV microarrays hybridized with cDNA of vAcIE1scmR-transduced Vero E6 cells harvested at different time points. (a) Time-course profiles of genes activated by IE1 in Vero E6 cells. (b) *gp64* transcript levels at different time points were compared with the levels of CMVie-promoter-driven *ie1*. (c) Western blot analysis of vAcIE1scmR- or vAscmR-transduced Vero E6 cells at different time points. The gp64 band can be clearly seen in the 48 and 72 h samples of the top panel as detected by an anti-gp64 antibody.

gp64 transcript were higher than that of *ie1* at 48, 72 and 96 h post-transduction. This result was double checked by Western blot analysis of total protein from transduced Vero E6 cells, probed with anti-*gp64* antibody. In both the 48 and 72 h samples, a clear gp64 protein band was seen in vAcIE1scmR-transduced Vero E6 cells (Fig. 4c). At the earlier time points, either the *gp64* transcripts had not been translated, or the amount of gp64 protein was below the level of detection. At the 96 h time point, the transduced cells were inactive due to either virus burden and/or confluence; the cellular translation mechanism was most likely not functional.

Identification of baculoviral genes activated by IE1 and IE2

Previously it was not known whether IE2 is functional in mammalian cells. Here, we found that although IE2 did not activate as many genes as IE1, both *pe38* and *orf17* genes transcripts were clearly seen on the IE2 microarray (Fig. 3c), their promoters activated by IE2 protein.

Surprisingly, when both vAcIE1scmR and vAcIE2scmR were used to transduce Vero E6 cells, the combined effects of the two *ie* genes were far greater than that of each individual gene. Around 38 % of the AcMNPV genome was clearly detectable on the IE1 + IE2 microarray (>180 F.U.) (Figs 3d, 5); the most intense gene spots (>1000 F.U.) were *gp64*, *orf25*, *orf17*, *he65*, *pe38*, *lef3*, *39K*, *pcna* and *orf16*. The values of all 59 activated genes are listed in Supplementary Table S2 (available in JGV Online). All the high level transcripts were from early genes or early and late genes, classified by their promoter sequences (Ayres *et al.*, 1994). Some of these genes, *he65*, *39K* and *gp64*, had been

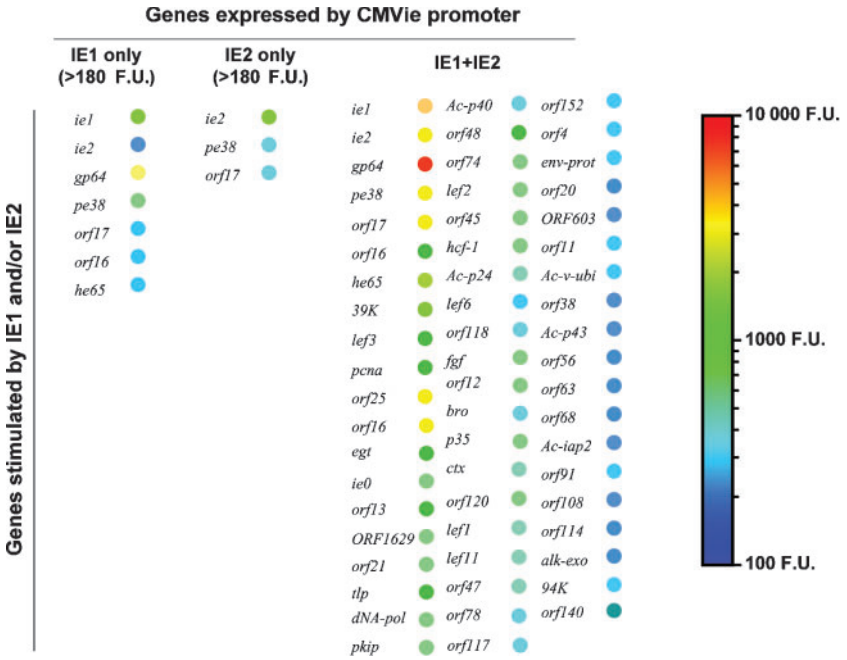

Fig. 5. Baculoviral genes induced by either IE1 and/or IE2 in Vero E6 cells at 48 h post-transduction. Six, two, and 59 baculovirus genes were induced in Vero E6 cells by IE1 and/or IE2, respectively. Only those genes that have expression levels over 180 F.U. (fluorescence units) are listed. The expression levels of viral genes generated by the transduction of the control vAscmR virus were all less than 180 F.U.

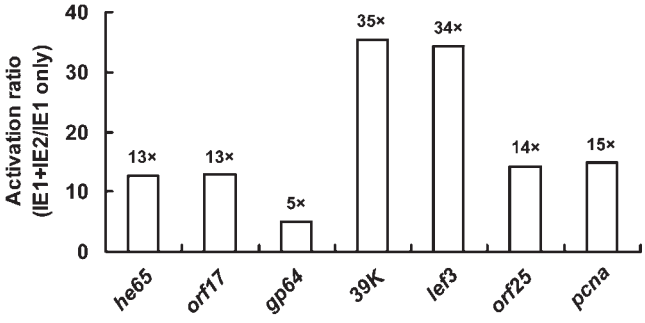

Fig. 6. Activation of AcMNPV early genes by IE1 plus IE2 were greater than IE1 alone. The fluorescence intensity of cy5-labelled transcripts from the IE1+IE2 microarray were divided by the results from the IE1 only microarray. Levels of *39K* and *lef3* gene transcriptions were noticeably greater when IE1 and IE2 were both present.

identified as IE1-activated genes by transient assays in insect cells (Blissard & Rohrmann, 1991; Guarino & Summers, 1986; Murges *et al.*, 1997). Interestingly, it is known that the *39K* gene requires both IE1 and IE2 activators for efficient activation in insect cells (Carson *et al.*, 1988). In Vero E6 cells, the level of *39K* transcript was found to be barely detectable on the IE1 microarray (63 F.U., 48 h post-transduction); however, it became highly abundant on the IE1+IE2 array (1485 F.U., 48 h post-transduction). The activation ratios of the combined IE1+IE2 *trans*-activators compared with IE1-only for *he65*, *orf17*, *gp64*, *39k*, *lef3*, *orf25* and *pcna* genes are shown in Fig. 6.

Promoter analysis of IE1-responsive genes

The group of genes activated by IE1 was either directly or indirectly turned on by the IE1 protein. In the case of a direct physical linkage, it is likely that an IE1-binding element could exist within the 5′ untranslated region (UTR) of the responsive genes. Bearing this in mind, the 5′ UTR of IE1-responsive genes up to position −160 were analysed using the web program Transcription Regulatory Element Search (*TRES*) (http://bioportal.bic.nus.edu.sg/tres/), as well as a group of non-IE1-responsive genes. Searching for conserved elements within this region, using a k-tuple size of eight allowing for one mismatch, revealed several conserved strings of bases, including a previously published IE1-binding element, ACBYGTAA (Leisy *et al.*, 1997). This element was found to exist both within the AcMNPV *hr* region, as well as the promoter regions of *ie0*, *ie2* and *pe38*. Of the more highly activated IE1 and IE2 genes (>1000 F.U.), the element ACTTGTAA with one allowed mismatch was found in the 5′ UTR of *pe38*, *he65*, *orf16*, *ie2* and *gp64* genes (Table 1). It was also found in the promoter region of *39K* and *ie1*, albeit on the complementary strand. Further testing is needed to confirm the significant role of these IE1-binding motifs to the promoter regulation of these genes.

Table 1. Properties of genes activated by IE1 and IE2 in Vero E6 cells

Gene	Transcriptional signals*	IE1-binding element (ACBYGTAA)†	TATA box (TATAAAAG)	Kozak rules‡
ie1	C, E	−47c	−121	+
ie2	C, E, L	−57	−80	−
gp64	C, E, L	−48	−69	+
pe38	E, L	−54	−75	+
orf17	E		−54	+
orf16	E	−152	−64	+
he65	C, L	−137 (in *hr*)	−42	+
39K	C	−14c	−158	+
lef3	C, E		−108	+
pcna	E		−71	+
orf25	C		−138	+

*The transcriptional signals C=CAGT and E=CGTGC; A(A/T)CGT(G/T) are motifs typical for early transcription. The L=TAAG represents the late transcription start signal (Ayres *et al.*, 1994).
†The number is relative to the ATG start signal where A=+1, the c after the number indicates the IE1-binding element is on the complementary strand (Leisy *et al.*, 1997).
‡A positive (+) symbol in the Kozak rule column indicates the gene contains the Kozak sequence [ANNATG(A/G); GNNATGG] surrounding the translation initiation site.

Transient assay analysis of IE1- and IE2-responsive genes in mammalian cells

Microarray studies gave us a global activation pattern by IE1 and IE2 *trans*-activators; however, it is hard to distinguish direct activation, through physical interactions between the activators and the promoters, from indirect activation, relying on the function of subsequent activators induced by IE1 or IE2 protein. The most direct analysis for interaction between a *trans*-activator and a promoter is transient expression assay. Reporter plasmids containing a baculoviral promoter driving a luciferase gene were used for IE1/IE2 gene activation studies (Fig. 7). Ten baculoviral promoters were tested for their ability to be activated by IE1 and IE2. These genes were discovered through microarray analysis to have a high to moderate level of transcription induced by IE1- or IE2-expressing recombinant baculoviruses. The results showed that IE1 protein can directly activate *ie1*, *ie2*, *pe38*, *39K*, *he65*, *pcna*, *orf16* and *orf25*, but not *gp64* or *orf17*. IE2 protein can weakly activate *he65* and *orf16*. Surprisingly, when in combination, IE1 and IE2 proteins in transient assay do not have a synergistic effect on gene activation. This result most likely indicated that the synergistic characteristic of IE1 plus IE2 activation involves other viral or cellular components, and this effect

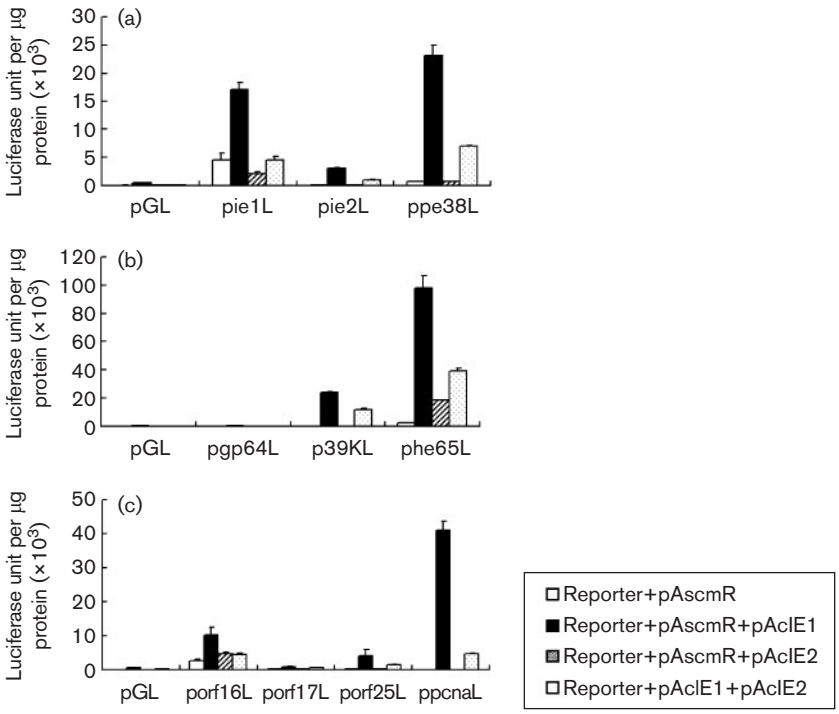

Fig. 7. Transient expression assays of the IE1 and/or IE2 induced gene promoters in Vero E6 cells. Effector plasmids pAcIE1, pAcIE2 or control plasmid pAscmR, either separately or in combination (equal amounts), were used in co-transfection assays with reporter luciferase plasmids. (a) The effects of IE1 or IE2 effector plasmids on the promoter of the three *ie* genes *ie1*, *ie2* and *pe38*. (b) The three previously known *ie1*-responsive genes, *gp64*, *39K* and *he65*, have different responses to IE1 and IE2 proteins in mammalian cells. (c) The effects of IE1 or IE2 effector plasmids on the promoter of the four AcMNPV early genes, *orf16*, *orf17*, *orf25* and *pcna*, not previously known to respond to IE1. The pGL plasmid is the control luciferase plasmid with no promoter in front of the luciferase ORF. Each sample was done in triplicate; the experiment was performed three times.

can only be seen *in vivo* in conjunction with the whole AcMNPV genome.

DISCUSSION

In this paper, we investigated the effects of two baculovirus *ie trans*-activators, IE1 and IE2, on AcMNPV genome activation in mammalian cells. By introducing IE1- and IE2-expressing recombinant baculoviruses into Vero E6 cells we were able to visualize their effects on AcMNPV transcriptome by using AcMNPV microarrays. When both IE1 and IE2 were present, around 38 % of AcMNPV genes were transcribed, indicating the two activators act synergistically in the context of AcMNPV genome. This finding has shone a new light on the nature of the strict host range specificity of baculovirus, and indeed shows many baculoviral promoters can function within mammalian cells, provided that the crucial transcription factors, e.g. IE1 and IE2, are present.

In Vero E6 cells, we optimized the baculovirus transduction dosage to an m.o.i. of 10. At this minimal inoculation ratio, we observed almost no endogenous viral gene transcription. A recently published AcMNPV DNA microarray study by Fujita *et al.* (2006) has shown low but detectable levels of *ie1* and *ie2* gene transcripts in BHK-21 cells and *ie1* transcript in HeLa14 cells when inoculated with AcMNPV at an m.o.i. of 30. The normally low endogenous expression level of baculoviral genes in mammalian cells, however, made microarray study of baculovirus gene regulation in mammalian systems difficult, as some transcripts may not be detectable above the background signal. In this study, a different approach was used where we selectively overexpressed specific baculoviral *trans*-activators and saw a significant induction of many viral genes in response to that.

A set of IE1-responsive genes were identified from the AcMNPV microarray hybridized with cDNAs from vAcIE1scmR-transduced Vero E6 cells, including *gp64*, *pe38*, *ie2*, *he65*, *orf16*, *orf17* and *orf25*. From previous transient transfection assays in insect systems, it had been shown that *gp64* and *he65* genes are positively regulated by IE1 (Blissard & Rohrmann, 1991; Murges *et al.*, 1997), while promoters of *ie2* and *pe38* contain an IE1-binding element (ACBYGTAA) and are negatively regulated (Leisy *et al.*, 1997). In mammalian cells, *ie2* and *pe38* gene expression was activated by IE1, and this discrepancy was probably because of host factor differences between the two systems. By analysing the promoters of IE1-responsive genes in Vero E6 cells, we identified several other IE1-responsive genes that also contain a sequence almost identical to this IE1-binding element (Table 1). It is highly probable that IE1 activated these genes by binding to this sequence.

Several other genes previously unlinked to IE1 were found to be activated by the vAcIE1scmR virus in the microarray experiment, including *pcna*, *orf16*, *orf17* and *orf25*. When all the vAcIE1scmR-virus-activated genes were tested by transient expression study in Vero E6 cells, two groups were found. One set of genes, *pcna*, *orf16* and *orf25*, was directly switched on by the IE1 protein, while the other set, *gp64* and *orf17*, was not. Because of the proximities of *orf16* and *orf17*, the possibility that the two gene signals on the microarray were from a single overlapping transcript could

not be overlooked. However, a closer look at the gene expression pattern of *orf16* and *orf17* (Fig. 4a) in a time-course study showed that the two adjacent genes on the same strand have different expression profiles. Therefore, it is unlikely that a single transcript was solely responsible for the signals on their corresponding spots on the microarray. The AcMNPV microarray contains unique gene fragments from each gene; care was taken to use a region of gene not overlapped with other genes on either strand. Also, gel-electrophoresis showed most cy3- or cy5-labelled cDNA samples were 0.5–1 kb in length, therefore only a small percentage of the signals should be from run-through transcripts. Nonetheless, the signals from adjacent genes oriented from the same strand should be viewed with caution; in this case, both time-course studies and transient expression assays were performed to support our microarray data.

The IE2-expressing recombinant baculovirus only weakly activated two genes on the microarray, *pe38* and *orf17*. Strikingly, in the presence of both vAcIE1scmR and vAcIE2scmR, both the number and intensity of the downstream genes increased significantly. Two likely reasons may be (i) the activated genes require the presence of both *ie* factors for activation, and/or (ii) IE1 and IE2 initiate a cascade of gene activation through subsequent transcriptional activators. The transient assay showed that a combination of IE1 and IE2 proteins has no obvious activation effect on the 10 baculoviral promoters. The mechanism of IE1+IE2 activation of AcMNPV genome is still unclear, but it obviously requires other viral factors/elements not present in a transient assay. For example, a distant *cis*-element could be missing in the reporter plasmid construct, thus rendering the *trans*-activators ineffective.

Protein expression experiments using baculovirus are currently predominantly performed in insect cells, due to a high yield. However, the differences in post-translational modifications between insect and mammalian cells are evident, and thus using mammalian cells as an alternative for protein expression experiments using baculovirus has been discussed in several studies (Boyce & Bucher, 1996; Hofmann *et al.*, 1995; Hu *et al.*, 2003; Shoji *et al.*, 1997). In this study, we found very late promoters were not directly activated by IE1 and IE2. However, an interesting finding was the strong activation of the AcMNPV *gp64* gene by the IE1-expressing recombinant baculovirus. The amount of *gp64* transcripts even surpassed *ie1* at 48 h post-transduction, even though *ie1* transcription was driven by the strong mammalian CMVie promoter.

The analysis of the AcMNPV transcriptome in Vero E6 cells in this study provided valuable information on baculovirus early gene regulation in the mammalian system. The IE1-activated genes in Vero E6 cells match well with ones found in insect cells, including *39K*, *he65* and *gp64*. In addition, the observation that *39K* gene activation required both IE1 and IE2 proteins agreed well with a previous study by Carson *et al.*(1988), where IE2 augmented IE1-induced *39K* gene activation in insect cells. The two systems, insect and mammalian, while evolutionarily distant, appear to have similar cellular machineries. Perhaps, given the right combination of baculoviral *trans*-activators, we could eventually utilize the extremely strong promoters of *p10* or *polyhedrin* for protein production in mammalian cells.

A promoter analysis of IE1/IE2-responsive genes showed all of them contained the baculovirus early transcription initiation sites C (CAGT) or E [CGTGC; A(A/T)CGT(G/T)] (Table 1). This concurred with our hypothesis that IE1/IE2 turns on other baculovirus genes in gene cascades similar to ones observed in insect cells. Most of these genes also contain a TATA motif (Table 1), which together with the partial eukaryotic transcriptional initiation site CAGT forms a promoter structure typical for eukaryotic RNA polymerase II. The involvement of IE1 or IE2 must be the last key step needed for these promoters to become active in mammalian cells.

Except for the *ie2* gene, all the major IE1- and IE2-responsive genes in Table 1 conform to Kozak rules [sequence: ANNATG(A/G) or GNNATGG; N=any nucleotide] (Kozak, 1987), while only 59 % of the genes in the AcMNPV genome do (Ayres *et al.*, 1994). Although a previous report indicated that the Kozak sequence does not affect yield of foreign protein expression in Sf21 cells when driven by the very late polyhedrin promoter (Hills & Crane-Robinson, 1995), our result suggests that a Kozak consensus sequence might be important for translation initiation of early baculoviral genes in mammalian cells.

In this study, we have attempted to map the interaction of some baculoviral genes, in detail, in a mammalian system. So far, by overexpressing IE1 and IE2, we have successfully turned on over a third of the baculoviral genome in cells that normally do not support baculoviral gene expression. Future experiments, involving expressing key late expression factor genes in mammalian cells, will hopefully enable us to activate late and very late promoters of AcMNPV. This approach may lead to a strong baculovirus expression system for mammalian cells, as well as give some insights into baculoviral gene regulation in its original host system.

ACKNOWLEDGEMENTS

We thank both S. Y. Tung and Y. H. Kao for their technical support on microarray analysis. This project was supported by grants from Academia Sinica (94S-1303) and National Science Council (NSC95-2313-B-001-031-MY3 and NSC95-2313-B-001-032-MY3).

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