Effects of Early or Overexpression of the *Autographa californica* Multiple Nucleopolyhedrovirus *orf94* (ODV-e25) on Virus Replication

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Abstract

*orf94* (ODV-e25) is one of the core genes of baculoviruses. To investigate how it functions in the replication cycle of a baculovirus, a number of *Autographa californica* multiple nucleopolyhedrovirus recombinants with e25 under control of the promoter of immediate early gene *ie1*, or the promoter of the very late hyperexpressed gene *p10*, were constructed using a bacmid system, and the effects of early expression or overexpression of e25 on replication of the virus were evaluated. Microscopy and titration assays demonstrated that bacmids with e25 under control of *ie1* promoter were unable to produce budded viruses; and that the recombinant viruses with e25 under control of *p10* promoter generated budded virus normally, but formation of occlusion bodies were dramatically reduced and delayed in the infected cells. Electron microscopy showed that there were no mature virions or intact nucleocapsids present in the cells transfected with a recombinant bacmid with e25 under control of *ie1* promoter. Quantitative real-time PCR analysis demonstrated that alteration of the e25 promoter did not affect viral DNA synthesis. The reporter gene expression from the promoter of the major capsid protein gene *vp39* was reduced 63% by early expression of e25. Confocal microscopy revealed that E25 was predominantly localized in nuclei by 24 hours post infection with wild-type virus, but it remained in the cytoplasm in the cells transfected with a recombinant bacmid with e25 under control of the *ie1* promoter, suggesting that the transport of E25 into nuclei was regulated in a specific and strict time dependent manner.

Introduction

*Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) belongs to the Baculoviridae. During the infection cycle, AcMNPV produces two types of virions: budded virus (BV) and occlusion derived virus (ODV), which are distinct in structure and function, and are responsible for the initiation of systematic infection within the body of a host insect and to spread infection to different members of susceptible insect species, respectively [1]. Both BV and ODV contain enveloped rod-shaped nucleocapsids that are assembled in the nucleus. In the early phase of infection, newly assembled nucleocapsids exit the nucleus and acquire an envelope by budding through the plasma membrane that is premodeled by viral proteins, producing mature BVs. After budding, BVs attach to other susceptible cells to initiate secondary infections [2,3]. In the late phase, nucleocapsids are enveloped by viral induced membranes within the nucleoplasm, forming ODVs, which are occluded in a protein crystal matrix, named occlusion bodies (OBs). Upon lysis of the infected cells, OBs are released into the environment. When OBs are consumed by another susceptible insect, the ODV virions are released to infect the midgut epithelial cells, initiating a new infection cycle [4]. BV and ODV share the same genome sequence, but differ in the composition of proteins associated with the envelope. The BV envelope contains several virally encoded proteins including GP64 that is a low pH activated envelope fusion protein that is required for entry of BV into cells [5,6]. In contrast, many of the ODV envelope-associated proteins differ from BV. ODV contains a group of proteins named per os infectivity factors required for oral infection and several other proteins [7–14]. In *Helicoverpa armigera* NPV (HearNPV), there are 12 BV-specific and 21 ODV-specific envelope proteins identified by comprehensive proteomics analyses [15].

ODV-E25 (E25) was originally identified as a 25 KD protein in *Orgia pseudotsugata* MNPV (OpMNPV) and AcMNPV, and was localized to the envelopes of ODV in OpMNPV [13]. Proteome analyses have shown that E25 is an ODV component in AcMNPV, HearNPV and *Chrysodeixis chalcites* NPV, and also a component of BV in AcMNPV [14,16–18], E25 of OpMNPV, AcMNPV and *Spodoptera litura* MNPV was detected in infected cells as doublets of about 25–26 KD and 27–28 KD, respectively [13,19]. AcMNPV E25 and several additional envelope proteins contain an N-terminal hydrophobic sequence in combination with several adjacent positively charged amino acids, which are predicted to be motifs that target these proteins to the nuclear envelope, intranuclear microvesicles and ODV envelopes [7,20]. The intranuclear microvesicles are thought to be precursors from which the envelopes of ODVs are derived. In AcMNPV, E25 is encoded by *orf94*, which has orthologs in the genomes of all baculoviruses sequenced to date and is considered a baculovirus protein.
“core gene” [21,22]. It was recently reported that AcMNPV e25 is required for budded virus infectivity and occlusion derived virus formation [23]. However, it is still unknown how E25 functions in viral replication.

Replication of AcMNPV and other baculoviruses proceeds through a series of well-ordered stages, which are administered by an expression cascade of the viral genes. The gene expression of the viruses can be divided into early, late and very late phases. Each gene has a specific time course of expression in virus replication cycle. Generally, genes encoding the proteins which are involved in viral DNA replication and/or late gene expression (eg. lef1-12) are expressed at an early time; structural protein genes (eg. gb39, p6.9 and e25) are expressed at late times; some genes are expressed at both early and late phases (eg. ie1, pp31 and gb64); p10 and polyhedrin (polh) are highly expressed very late genes, which are expressed through late and very late times of infection [24]. Although many reports have described investigations of the effects of baculovirus gene knockouts on the virus life cycle, few have examined the effects of altering the temporal or elevated expression of essential genes. The altered temporal expression of an essential core gene, such as e25, could have an impact on virus replication by disrupting the normal progression of molecular events. Hence, it is possible to investigate the role of a viral gene from phenotypic variations induced by temporal changes in its expression. In this study, the effects of early expression or very late overexpression of e25 on the replication of AcMNPV was investigated. It was found that early expression of e25 severely disrupted both BV and ODV production. Although the overexpression of e25 did not have significant effects on BV production or assembly of virions, it inhibited the formation of occlusion bodies.

Results
Generation of recombinant AcMNPV bacmids with e25 under control of alternative promoters

To determine the effect of the changes in the time course of expression of e25 on virus replication, several recombinant bacmids were constructed, in which the original egfp was deleted and another copy of egfp with an alternative promoter was inserted back into the bacmids at the polh locus. At first, an e25 knockout bacmid, vAcE25ko, was constructed, in which the 3’-end of the e25 ORF (nt2-592) was deleted and replaced with the chloramphenicol acetyltransferase (cat) gene facilitating antibiotic selection in E. coli (Fig. 1A & B). Two bacmids designed to express e25 in early phase or over-express the protein in very late phase under control of alternative promoters replicated in the transfected cell cultures and monitored by fluorescence and phase contrast microscope. As shown in Fig. 2A, fluorescence was first observed in cells transfected with all the five individual bacmids, at 24 h.p.t. No obvious difference in number and lightness of the fluorescent cells was observed. At 96 h.p.t., the majority of the cells in the dishes containing vAcPH-gfp, vAcPp10-e25-PH-gfp, or vAcPp10-egfp-PH-gfp, were fluorescent, whereas there was no significant increase in the number of fluorescent cells with vAcPie1-e25-PH-gfp or vAcE25ko-PH-gfp, suggesting that the spread of infection occurred in the cells with wt, e25-knockout repair, or the bacmid with e25 under control of the polh promoter; but not in the cells transfected with the bacmid with e25 deleted or the one with e25 driven by the ie1 promoter.

At 120 h.p.t., supernatants were collected from the dishes inoculated with the supernatants from the transfection with vAcPH-gfp, vAce25ko-rep-PH-gfp and vAce25ko-PH-gfp were used as controls. Cells were then incubated with supernatants from transfected cell cultures and monitored by fluorescence and phase contrast microscope. As shown in Fig. 2B, fluorescence was first observed in cells transfected with all the five individual bacmids, at 24 h.p.t. No obvious difference in number and lightness of the fluorescent cells was observed. At 96 h.p.t., the majority of the cells in the dishes containing vAcPH-gfp, vAcPp10-e25-PH-gfp, or vAcPp10-egfp-PH-gfp, were fluorescent, whereas there was no significant increase in the number of fluorescent cells with vAcPie1-e25-PH-gfp or vAcE25ko-PH-gfp, suggesting that the spread of infection occurred in the cells with wt, e25-knockout repair, or the bacmid with e25 under control of the polh promoter; but not in the cells transfected with the bacmid with e25 deleted or the one with e25 driven by the ie1 promoter.

The effects of early or late overexpression of e25 on virus replication were further examined by a virus growth curve analyses. As shown in Fig. 2C, the virus titers from the cell culture transfected with vAcPie1-e25-PH-gfp, vAcPp10-e25-PH-gfp, or vAcPp10-egfp-PH-gfp, were less than 1020 h.p.i., whereas Sf9 cells infected with vAcPie1-e25-PH-gfp revealed a steady increase in virus production, similar to the cells infected with vAcE25ko-egfp-PH-gfp. These results indicate that early expression of the e25 blocks production of infectious budded virus, whereas overexpression of the gene driven by the polh promoter does not have significant effects on the BV replication of the AcMNPV.

Under phase contrast microscopy, OBs were seen in the cells transfected individually with vAcPH-gfp, vAcE25ko-egfp-PH-gfp, vAcPie1-e25-PH-gfp and vAcE25ko-PH-gfp at late times, but few OB-containing cells and no evidence of the spreading of the infection was observed in the transfections with vAcPie1-e25-PH-gfp or vAcE25ko-PH-gfp, [data not shown]. To eliminate potential effects from the extra gb16 promoter and the egfp sequence inserted at the same locus, three additional bacmids vAcE25ko-egfp-PH-gfp, vAcPie1-e25-PH-gfp and vAcPp10-e25-PH-gfp were constructed (Fig. 1B). OBs were first observed at 48 h.p.t. in the cells transfected with vAcE25ko-egfp-PH-gfp and the cells with vAcPie1-e25-PH-gfp. By 96 h.p.t., the majority of cells

Two additional bacmids vAcPie1-e25-PH-gfp (Fig. 1A) and vAcE25ko-egfp-PH-gfp (Fig. 1B) were constructed by inserting a copy of the polh and egfp into the AcMNPV bacmid bMON14272 or vAcE25ko. They were respectively used as wild-type (wt) and e25-negative controls in this study.

Effects of early/over expression of the e25 on virus production

To examine the effects of early expression or overexpression of the e25 gene on virus replication, the bacmids vAcPie1-e25-PH-gfp and vAcPp10-e25-PH-gfp were separately transfected into Sf9 cells. vAcPH-gfp, vAcE25ko-egfp-PH-gfp and vAcPie1-e25-PH-gfp were used as controls. Cells were then incubated with supernatants from transfected cell cultures and monitored by fluorescence and phase contrast microscope. As shown in Fig. 2A, fluorescence was first observed in cells transfected with all the five individual bacmids, at 24 h.p.t. No obvious difference in number and lightness of the fluorescent cells was observed. At 96 h.p.t., the majority of the cells in the dishes containing vAcPH-gfp, vAcPp10-e25-PH-gfp, or vAcPp10-egfp-PH-gfp, were fluorescent, whereas there was no significant increase in the number of fluorescent cells with vAcPie1-e25-PH-gfp or vAcE25ko-PH-gfp, suggesting that the spread of infection occurred in the cells with wt, e25-knockout repair, or the bacmid with e25 under control of the polh promoter; but not in the cells transfected with the bacmid with e25 deleted or the one with e25 driven by the ie1 promoter.
Figure 1. Construction of recombinant AcMNPV bacmids. (A) Schematic map of the structures of the orf94 (e25) locus and the polh locus in a wt AcMNPV bacmid vAcPH-gfp. A copy of egfp ORF under control of AcMNPV gp16 promoter and a copy of polh were inserted into the polh locus in opposite orientation. (B) Schematic maps of the structures of the e25 locus and the polh locus in e25 knockout and repair bacmids. In e25 locus, a 591 bp sequence of the orf94 was deleted and replaced with the cat. In polh locus, a copy of egfp ORF under control of the gp16 promoter and a polh were inserted into the polh locus in opposite orientation (vAce25ko-PH-gfp). Alternatively, a polh with native promoter and an e25 with native promoter (vAcPie1-e25ko-PH) or p10 promoter (vAcPp10-e25ko-PH) was inserted in the polh locus in opposite orientation. (C) Time course analysis of the E25 expressed in Sf9 cells infected by vAcPie1-e25-PH-gfp, vAcPp10-e25-PH-gfp or vAc25ko-rep-PH-gfp. The cells transfected with the individual bacmids were harvested at designated time points post transfection, and the cell extracts subjected to SDS-PAGE, and immunoblot analysis with E25-specific antiserum. doi:10.1371/journal.pone.0065635.g001
Figure 2. Analysis of viral replication in Sf9 cells transfected/infected with AcMNPV recombinants with e25 under control of alternative promoters. (A) Fluorescence microscopy of Sf9 cells transfected with vAc\textsuperscript{PH-gfp}, vAc\textsuperscript{e25ko-PH-gfp}, vAc\textsuperscript{Pie1-e25-PH-gfp}, vAc\textsuperscript{Pp10-e25-PH-gfp} or vAc\textsuperscript{e25ko-rep-PH-gfp}, at 24 and 96 h.p.t. (B) Fluorescence microscopy of Sf9 cells infected with the supernatants from transfections above, at 24 and 72 h.p.i. (C) Virus growth curves of vAc\textsuperscript{Pie1-e25-PH-gfp}, vAc\textsuperscript{Pp10-e25-PH-gfp} and vAc\textsuperscript{e25ko-rep-PH-gfp} in Sf9 cells. Sf9 cells were inoculated with the supernatant from the cell cultures transfected by vAc\textsuperscript{Pp10-e25-PH-gfp} or vAc\textsuperscript{e25ko-rep-PH-gfp} at a MOI of 5, or 1000 μl of the supernatant from the cell culture.
transfected by vAcP<sub>e25ko-rep-PH</sub> were filled with OBs. In contrast, OBs were observed in only a few isolated cells in the cultures transfected with vAcP<sub>e1-25-PH</sub>. In the cells transfected with vAcP<sub>P10ko-e25-PH</sub>, OBs were occasionally found in few cells by 120 h.p.t. (Fig. 2D).

Effects of early expression or late overexpression of e25 on virus morphogenesis

To further determine if temporal alteration in expression of e25 had any effect on virus morphogenesis, electron microscopic analysis was performed with thin sections of the cells transfected with vAcP<sub>P10ko-e25-PH</sub>, vAcP<sub>P10-e25-PH</sub> or vAcP<sub>e25ko-rep-PH</sub>. At 96 h.p.t., the cells transfected with vAcP<sub>e25ko-rep-PH</sub> showed the typical characteristics of a baculovirus infection. Virogenic stroma inundated with rod-shaped nucleocapsids (Fig. 3A) and nucleocapsids acquiring their envelopes and embedding into the developing OBs (Fig. 3B) were observed. Similarly, virogenic stroma (Fig. 3C), abundant enveloped nucleocapsids (Fig. 3D) within enlarged nuclei and single nucleocapsids budding through cytoplasmic membrane could also be observed (Fig. 3E) in the cells transfected with vAcP<sub>P10-e25-PH</sub>, but OBs were not found, obviously due to their rareness (as shown in Fig. 2D). In the cells transfected with vAcP<sub>P10-e25-PH</sub> and vAcP<sub>P10ko-e25-PH</sub>, virogenic stroma-like structures could be observed (Fig. 3F), but there were not any mature nucleocapsids present. Only a few rod-shaped empty capsids were observed in the nuclei (Fig. 3G & H). The electron microscopy indicated that whereas early expression of e25 interfered with nucleocapsid assembly, late overexpression of e25 had no effect on nucleocapsid assembly, but interfered with OB formation.

Effects of deletion or early expression of the e25 on virus DNA replication

The levels of viral DNA replication in the cells transfected individually with vAcP<sub>e25ko-ph</sub> and vAcP<sub>P10-e25-PH-gfp</sub> were measured over a 120 h time-course, to determine if e25 had an impact on viral DNA replication. The transfected Sf9 cells were collected at designated time-points, and the total DNA was extracted and analyzed by qPCR. vAcP<sub>e25ko-ph</sub>, which is a gfp<sub>64</sub>-knockout mutant of the wild type bacmid BMON14272 was used as a control. For all three viruses, DNA synthesis began increasing at 12 h.p.t. and continued until 72 h.p.t. (Fig. 4). The levels of DNA detected for vAcP<sub>e25ko-ph</sub> were higher than for vAcP<sub>P10-e25-PH-gfp</sub> and vAcP<sub>e25ko-ph</sub> before 72 h.p.t. The levels of DNA detected for vAcP<sub>e25ko-ph</sub> were higher than for vAcP<sub>P10-e25-PH-gfp</sub> and vAcP<sub>e25ko-ph</sub> before 72 h.p.t. However, the peak levels reached by all the three bacmids at 72 h.p.t. were similar. These results indicated that the total level of replication in individual infected cells was unaffected by deletion or early expression of e25, although the DNA replication might be accelerated slightly by the mutations. This could also be due to the lack of BV production, which would cause the DNA to accumulate in the KO, and P<sub>e1</sub>-e25 cells.

Early expression of the e25 knocks down gus expression driven by vp39 promoter

Effects of early expression of the e25 on virus gene expression were also evaluated by assays using a B-g-glucuronidase (GUS) gene under control of the vp39 promoter. vp39 encodes the major capsid protein. It is expressed in late phase in infection [27].

Three late expression reporter bacmids vAcP<sub>e25ko-Pvp39-gus</sub>, vAcP<sub>P10-e25-Pvp39-gus</sub>, and vAcP<sub>e25ko-rep-PH</sub>, which contain the gus under control of a vp39 promoter, were constructed (Fig. 5A). All of the reporter bacmids have the original e25 deleted. vAcP<sub>P10-e25-Pvp39-gus</sub> and vAcP<sub>e25ko-Pvp39-gus</sub> have a copy of e25 under control of an ie1 promoter and an e25 with the native promoter inserted at the polh locus respectively. Sf9 cells transfected with individual reporter bacmids were collected at designated time points and used for GUS assays.

GUS activity was first detected at 24 h.p.t. in all cases. The levels of GUS activity detected in extracts of the cells transfected with vAcP<sub>e25ko-Pvp39-gus</sub> were similar to the ones of the cells transfected with vAcP<sub>e25-Pvp39-gus</sub> at all time points up to 48 h.p.t. However, GUS activity was significantly lower in the cells transfected with vAcP<sub>P10-e25-Pvp39-gus</sub> than the ones in the cells transfected with the two other reporter bacmids, being 64% and 63% lower than the GUS activities in the cells transfected with vAcP<sub>e25-Pvp39-gus</sub> at 36 and 48 h.p.t. respectively (Fig. 5B). These results suggest that deletion of e25 has minimum effects on, but early expression of e25, reduces late gene expression driven by the vp39 promoter.

Localization of E25 in AcMNPV-infected insect cells

Subcellular localization of E25 in infected Sf9 cells was analyzed by immunofluorescence microscopy in combination with nuclear staining by Hoechst33258. Sf9 cells were infected with vAcP<sub>PH-gfp</sub> at a MOI of 5. At designated time points, the cells were sampled, blotted with E25-specific antiserum and Rhodamine-conjugated goat-anti-rabbit IgG, stained with Hoechst33258, and subjected to confocal microscopy.

The E25 labeled by Rhodamine with red fluorescence was first observed predominantly in the cytoplasm at 12 h.p.i. (Fig. 6). At 18 h.p.i., about half of the red fluorescence was present in nuclei (blue color) in dot like structures. By 24 h.p.i., red fluorescence could only be observed in nuclei, forming a ring zone at periphery of the nucleus. By 72 h.p.i., red fluorescence spread throughout the nucleus (Fig. 6).

Early expression of e25 blocks nuclear transporting of E25

Localization of E25 in the Sf9 cells transfected by vAcP<sub>P10-e25-PH-gfp</sub> was analyzed by immunofluorescence microscopy in the similar way as mentioned above.

The E25 expressed by ie1 promoter was observed first in the cytoplasm at 12 h.p.i., as red fluorescence emitted by Rhodamine-conjugated goat-anti-rabbit IgG, which was associated with E25 through E25-specific antibodies (Fig. 7). No red fluorescence signal was found in the nuclei until 24 h.p.t. At 48 h.p.t., a small region of red fluorescence was observed in the nuclei, but the density of the red fluorescence did not increase by 72 h.p.t., most still remained in the cytoplasm. In contrast, when expressed from its own promoter, E25 was predominantly localized in the nucleus by 48 h.p.t. (Fig. 7). This phenomenon demonstrated that the early expression of E25 prevented the trafficking E25 into the nucleus in a transfected cell.

Discussion

The infection cycle of AcMNPV and other baculoviruses is organized by a complex transcriptional cascade. Early genes are
expressed prior to DNA replication and are transcribed by host cell RNA polymerase II [28,29]. Late and very-late genes are transcribed following the onset of DNA replication by a virus-encoded RNA polymerase [28,30–32]. The mechanism controlling the transition from early gene expression and DNA replication to late gene expression is not clear.

*e25* is expressed at the late phase in natural replication cycles of AcMNPV and locates in the nuclei of the infected cells [13]. To determine effects of the temporal change of expression of *e25* on virus replication, AcMNPV recombinants expressing E25 under control of the promoter of the immediate early gene *ie1* or the promoter of the overexpressed very late gene *p10*, were constructed in this study; and the phenotypic variations induced by the temporal changes of expression of *e25* were analyzed. We found that early expression of *e25* almost completely eliminated production of infectious BV (Fig. 2). In addition, in the cells transfected with the bacmids in which the *e25* was placed under control of the *ie1* promoter, no mature virions or intact nucleocapsids were observed (Fig. 3). In contrast, overexpression of *e25* did not cause significant change in the production, infectivity of BV, or assembly of virions in the nuclei of infected cells.

To explore the mechanism behind the phenotypic variations resulting from the early expression of *e25*, the effects on viral DNA replication and late gene expression were evaluated by Q-PCR and by transient assays. It was shown that early expression of *e25* did not affect viral DNA replication (Fig. 4), but resulted in a drop of 63% in expression level of the reporter gene driven by the *vp39* promoter (Fig. 5). Reduction in expression of some late genes could affect virus production. How the early expression of *e25* results in reduction of another late gene remains to be elucidated.

Since ODVs are assembled in nuclei of infected cells, all structural proteins have to translocate from the site they are synthesized in cytoplasm into nuclei. It has been shown that the ODV envelope proteins traffic through the ER, outer nuclear membrane, inner nuclear membrane, and nuclear pore complex, which is a continuous network of membranes [33]. To test if early expression of *e25* affects the transport of E25 into nuclei, the subcellular localization of E25 in the cells transfected with the bacmid with *e25* under control of *ie1* promoter were tracked by immunofluorescence assays, and compared with the cells transfected with the *e25* knockout repair bacmid. As a result, E25 in the cells transfected by vAc<sup>Pie1-e25-PH-gfp</sup> mostly remained in the cytoplasm until 72 h.p.t., in contrast to the cells transfected by

![Figure 3. Transmission electron microscopy analysis of Sf9 cells transfected with vAc<sup>e25ko-rep-PH-gfp</sup> (A and B), vAc<sup>Pp10-e25-PH-gfp</sup> (C-E), or vAc<sup>ie-e25-PH-gfp</sup> (F-H), at 96 h. p.t. (A) Nucleocapsids (Nu) present around the virogenic stroma in an enlarged nucleus (VS). (B) Virions embedded in an occlusion body (OB). (C) Virogenic stroma with a few nucleocapsids associated. (D) Enveloped nucleocapsids. (E) Virions budding through cytoplasmic membrane. (F) Virogenic stroma-like structure. (G) Two rod-shaped nucleocapsid-like particles present in an enlarged nucleus. (H) Empty rod-shaped capsids (Cp). Scale bar = 500 nm. doi:10.1371/journal.pone.0065635.g003](http://www.plosone.org/FIG3.jpg)
vAc e25ko-rep-PH-gfp where E25 was almost completely localized in the nuclei by 24 h.p.t. This indicates that transport of E25 into nuclei was regulated in a time specific manner, in infected cells. E25 was previously shown to interact with ODV-E66, which also bound to FP25K and BV/ODV-E26 [34]. FP25K and BV/ODV-E26 are involved in trafficking of viral envelope proteins [33,35]. Early expression of E25 may cause changes in interactions between E25 and other viral or host proteins or in modifications on E25, that subsequently interrupt trafficking of E25 into the nucleus. If E25 is involved in envelopment of ODV, as proposed [10], blocking of transporting E25 into nuclei would affect ODV envelopment that occurs in nuclei. This is evidenced by the observation from electron microscopy of the cells transfected with vAcPie1-e25-PH, where there were no enveloped virions present. It was previously reported that there were no virions found in the cells transfected with a recombinant AcMNPV bacmid with e25 deleted [23]. However, the early expression of E25 suggests that it is involved in the proper assembly of nucleocapsids independent of its role in the ODV envelope. The early expression of E25 could alter DNA packaging by binding to and preventing the assembly of a component of the packaging complex that it normally interacts with later in infection. This could account for the apparent lack of DNA in the nucleocapsids, although it remains to be proven by additional experimental evidence.

To date, more than twenty proteins have been identified to be associated with envelopes of ODV and/or BV in AcMNPV and other baculoviruses. E25 is among a few envelope proteins present in both ODV and BV. BVs acquire their envelopes from modified plasma membrane whereas ODVs are thought to obtain theirs from intranuclear membranes. This suggests that E25 must be partitioned between both the cytoplasm and nucleus during virus replication. Although great progress have been made in deciphering the pathway of the ODV envelope proteins from their site of insertion into the membrane of ER through their transit to the inner nuclear membrane [7,33,36], the mechanism directing these proteins into nuclei or onto the plasma membrane remains to be determined.

In this study, the effects of very late overexpression of e25 on replication of AcMNPV were also evaluated. In cells infected with the recombinant virus with e25 placed under control of the p10 promoter, the production of BV was not affected by overexpression of e25, in comparison with the cells infected by the virus containing e25 with native promoter, but OB production was limited and they were found only in a few cells in very late phase in infection (Fig. 2). This result demonstrates that redundant E25 in the nuclei may inhibit occlusion of virions and formation of OB. However, it could also result from competition from the extra p10 promoter added at the polyhedrin locus. It was previously reported that competition between baculovirus polyhedrin and p10 gene expression occurred during infection of insect cells [37]. In this study, the polyhedrin detected in the cells infected with vAcPy10-e25-PH-gfp was less than that in the cells with vAcPy10-e25-PH-gfp (data not shown). Reduction of polyhedrin in the infected cells could affect formation of OBs.

Materials and Methods

Virus, cell line and primers

The AcMNPV bacmid bMON14272 was maintained in DH10B cells as described previously [38]. The Sf9 cell line (Invitrogen), a clonal isolate of the parent cell line IPLB-Sf21-AE from the fall armyworm Spodoptera frugiperda [39] were cultured at 27°C in Grace’s medium supplemented with 10% fetal bovine serum, penicillin and streptomycin.

The DNA primers used in this study were synthesized by GenScript, Inc. and are shown in Table 1.

Construction of e25 knockout AcMNPV bacmid

The AcMNPV bacmid bMON14272 was used to generate an e25 KO virus by recombination in E. coli using the λ Red system, as previously described [40]. A DNA fragment (DS) corresponding to the 3′-end (nt 80563–80578) of AcMNPV e25 was amplified by PCR with the primers dhUP and dhDP, and inserted into the PstI and HindIII sites of pUC19. The cat was amplified with the

Figure 4. Quantitative-PCR analysis of viral DNA replication in Sf9 cells transfected by recombinant AcMNPV bacmids with e25 deleted or under control of ie1 promoter. Total DNA was purified from the cells transfected with vAc e25ko, vAcP10-e25-PH-gfp, or vAc gp64ko, at 0, 12, 24, 48, 72, 96 and 120 h.p.t., digested with DpnI to eliminate input bacmid DNA, and analyzed by real-time PCR. The values displayed represent the averages from transfections performed in triplicate with error bars indicating standard deviations.

doi:10.1371/journal.pone.0065635.g004
primers catUP and catDP and inserted into the BamHI and PstI sites of the resultant plasmid. Then, the cat-DS fragment was isolated and inserted into the BamHI and HindIII sites of pBluescript II KS (-); and another DNA fragment (US) corresponding to the upstream sequence of e25 (nt 79492–79971) was amplified with the primers uhUP and uhDP, and inserted upstream the cat gene. The resultant plasmid was cut with XbaI and HindIII to isolate the US-cat-DS segment, which was electro-transformed into arabinose-induced E. coli DH10B cells harboring bMON14272 and pKD46 encoding l-Red recombinase. The resultant bacmid was named vAce25ko (Fig. 1B). Four sets of primers, uhUP/dhDP, uhUP/catDP, catUP/dhDP and catUP/catDP were used in PCR to confirm the proper replacement of e25 with cat cassette in the bacmid.

Construction of e25 knockout, repair, and wt AcMNPV bacmids containing egfp and/or polh

A DNA fragment containing AcMNPV polh with native promoter was PCR-amplified using the primers polhUP and polhDP. It was inserted between the EcoRI and BstZ17I sites of pFastBac1 (Invitrogen) to obtain pFB-PH. Another fragment containing egfp under control of an AcMNPV gp16 promoter was amplified with the primers Pgp16UP and gfpDP, using a plasmid pFB-Pgp16-gfp (unpublished) as template. It was inserted into the XbaI and XhoI sites of pFB-PH to produce pFB-PH-Pgp16-gfp.
pFB-PH-Pgp16-gfp was electroporated into E. coli DH10B containing bMON14272 and the helper plasmid pMON7124 [38] to generate a polh- and egfp-containing wt bacmid vAcPH-gfp (Fig. 1A). pFB-PH-Pgp16-gfp was electroporated into E. coli DH10B containing vAce25ko and pMON7124 to generate a polh- and egfp-containing e25-null bacmid vAc<sup>e25ko</sup>-PH-gfp (Fig. 1B).

A fragment containing e25 with the native promoter (nt79729–80868) was amplified using the primer pair c25UP/c25DP. It was inserted into the StuI site of pFB-PH-Pgp16-gfp to generate pFB-PH-c25-Pgp16-gfp. Another fragment containing e25 with the native promoter was amplified using the primers c25UP3 and c25DP3, and inserted between the SacI and BamHI sites of pFastBac1 to produce pFB-e25. A fragment containing polh (nt4300–5257) was amplified with the primer pair polhUP3/polhDP2, and inserted into the Xbal and SphI sites of pFB-c25 to obtain pFB-c25-PH. pFB-PH-c25-Pgp16-gfp and pFB-c25-PH was electroporated into E. coli DH10B containing vAc<sup>e25ko</sup> and pMON7124 to generate two e25-repaired bacmids, vAc<sup>e25ko-rep-PH-gfp</sup> and vAc<sup>e25ko-rep-PH</sup> respectively (Fig. 1B).

Figure 6. Subcellular localization of the E25 in Sf9 cells infected by AcMNPV. Sf9 cells infected by AcMNPV were sampled at 12, 18, 24, 48 and 72 h.p.i., blotted with E25-specific polyclonal antibodies which were subsequently blotted by using Rhodamine-conjugated goat-anti-rabbit IgG to label E25 (red), stained with Hoechst33258 to mark nuclei (blue), and subjected to confocal microscopy. doi:10.1371/journal.pone.0065635.g006

Figure 7. Subcellular localization of E25 in Sf9 cells infected by AcMNPV mutant with e25 under control of early gene promoter. The cells transfected by vAc<sup>Pie1-e25-PH-gfp</sup> were sampled at 12, 24, 48 and 72 h.p.t., blotted with E25-specific polyclonal antibodies which were subsequently blotted by using Rhodamine-conjugated goat-anti-rabbit IgG to label E25 (red), stained with Hoechst33258 to mark nuclei (blue), and subjected to confocal microscopy. Sf9 cells transfected by vAc<sup>e25ko-rep-PH-gfp</sup>, which were sampled at 48 h.p.t. and treated in the same way, were shown as control. doi:10.1371/journal.pone.0065635.g007

Construction of recombinant bacmids with e25 under control of alternative promoters

A fragment containing e25 ORF and transcription terminator was amplified with the primer pair e25UP2/e25DP2, and ligated with pUC19 cut with SacI and BamHI, producing pUC-e25. Another fragment containing an AcMNPV ie1 promoter (700 bp) was amplified with the primers Pie1UP and Pie1DP, and inserted into the HindIII and BamHI sites of pUC-e25, resulting in pUC-Pie1-e25. Using pUC-Pie1-e25 as template, a fragment containing the e25 under control of the ie1 promoter was amplified with primers Pie1UP2 and e25DP2, and ligated with pFB-PH-Pgp16-gfp cut with Stul and Sacl, to produce pFB-Pie1-e25. In the same way, a fragment containing AcMNPV p10 promoter (297 bp) was amplified with the primers Pp10UP and Pp10DP and inserted upstream of the e25 ORF of pUC-e25 to produce pUC-Pp10-e25, which was used as template to amplify a fragment containing e25 linked with a p10 promoter with the primers Pp10UP2/c25DP2. The PCR fragment was inserted between the Stul and Sacl sites of pFB-PH-Pgp16-gfp to construct pFB-Pp10-e25.
Table 1. Oligonucleotides used to generate knockout and repair bacmid constructs.

| Name            | Sequence                                      |
|-----------------|-----------------------------------------------|
| dhUP            | `5'CGGCTCTGCGATCCAGGACAAATTTTATC3`            |
| dhDP            | `5'CGTAAAGCTGACCCGATCTTCG3`                   |
| catUP           | `5'CGGCCGATCTGCGATACCAAAATTTTATC3`           |
| catDP           | `5'CTGACTCGAAGGACAAATTTTATC3`                 |
| uhUP            | `5'CGGCTGAGTACCGAATCGTGGTATGGTATG3`          |
| uhDP            | `5'GGCGGCGGATCTGCGATACCAAAATTTTATC3`         |
| polhUP3         | `5'AAAGGATTATATACGAGATCAATGACG3`             |
| polhDP3         | `5'AGAGGATTATATACGAGATCAATGACG3`             |
| polhUP2         | `5'AAAGGATTATATACGAGATCAATGACG3`             |
| polhDP2         | `5'AGAGGATTATATACGAGATCAATGACG3`             |
| Pie1UP3         | `5'GCAAACCTGAGTACCGATCCAGT3`                  |
| Pie1DP          | `5'ATGGAATACGATACGATCTGGTATG3`               |
| Pie1UP2         | `5'GAGAGGCTGCGATCGATCCAGT3`                   |
| Pie1UP3         | `5'GAACTCGGAGCTGCGATCCAGT3`                   |
| Pgp16UP         | `5'GAGAGGCTGCGATCGATCCAGT3`                   |
| e25UP           | `5'TAGAAGGCTTGGTGAATACCAAG3`                  |
| e25DP           | `5'CGGCTGAGTACCGAATCGTGGTATGGTATG3`          |
| e25UP2          | `5'TAGAAGGCTTGGTGAATACCAAG3`                  |
| e25DP2          | `5'GAGAGGCTGCGATCGATCCAGT3`                   |
| Pie1UP          | `5'GCAAACCTGAGTACCGATCCAGT3`                  |
| Pie1DP          | `5'ATGGAATACGATACGATCTGGTATG3`               |
| Pie1UP2         | `5'GAGAGGCTGCGATCGATCCAGT3`                   |
| Pie1UP3         | `5'GAACTCGGAGCTGCGATCCAGT3`                   |
| Pvp39UP         | `5'GCTGACTGAGATGATGCAGT3`                    |
| Pvp39DP         | `5'GCTGACTGAGATGATGCAGT3`                    |
| Pvp39UP2        | `5'GCTGACTGAGATGATGCAGT3`                    |
| Pvp39DP2        | `5'GCTGACTGAGATGATGCAGT3`                    |
| Pvp39UP3        | `5'GCTGACTGAGATGATGCAGT3`                    |
| Pvp39DP3        | `5'GCTGACTGAGATGATGCAGT3`                    |
| Pie13UP         | `5'GAACTCGGAGCTGCGATCCAGT3`                   |
| Pie13DP         | `5'GAACTCGGAGCTGCGATCCAGT3`                   |
| polhUP2         | `5'GAGAGGCTGCGATCGATCCAGT3`                   |
| polhDP2         | `5'GAGAGGCTGCGATCGATCCAGT3`                   |
| polhUP3         | `5'GAGAGGCTGCGATCGATCCAGT3`                   |
| polhDP3         | `5'GAGAGGCTGCGATCGATCCAGT3`                   |
| M13F            | `5'GTTTTCACCAAGCAAG3`                        |
| M13R            | `5'ATGGAATACGATACGATCTGGTATG3`               |
| e25UP4          | `5'GGGCTCTGCTGATGATGCAGT3`                    |

Sequences of restriction sites are underlined.

doi:10.1371/journal.pone.0065635.t001

A fragment containing the polh (nt4355-5257) was amplified with the primers polhUP2 and polhDP2, and inserted into the PsiI and SphI sites of pFastBac1 to obtain pFB-PH-2. Another fragment containing e25 under control of p10 promoter was amplified from pFB-P10-e25 with the primers P10UP3 and e25DP2, and inserted between the XbaI and SacI sites of pFastBac1 to construct pFB-P10-e25-PH. Using the primer pairs P10UP3 and e25DP2, and inserted between the SacI and SnaBI sites of pFastBac1 to make pFB-P10-e25. Another fragment containing polh (nt4300-5257) was amplified with primers polhUP3 and polhDP3 and inserted between the XbaI and PstI sites of pFB-P10-e25, producing pFB-P10-e25-PH. 

The primers used for PCR are listed in Table 1. The GUS coding sequence was amplified with the primers gusUP and gusDP. The reporter plasmid pCALL4 [41] was used as template, and a fragment containing GUS coding sequence was amplified with the primer pairs gusUP and gusDP. 


doi:10.1371/journal.pone.0065635.t001

Table 1. Oligonucleotides used to generate knockout and repair bacmid constructs.

| Name   | Sequence                                      |
|--------|-----------------------------------------------|
| dhUP   | `5'CGGCTCTGCGATCCAGGACAAATTTTATC3`            |
| dhDP   | `5'CGTAAAGCTGACCCGATCTTCG3`                   |
| catUP  | `5'CGGCCGATCTGCGATACCAAAATTTTATC3`           |
| catDP  | `5'CTGACTCGAAGGACAAATTTTATC3`                 |
| uhUP   | `5'CGGCTGAGTACCGAATCGTGGTATGGTATG3`          |
| uhDP   | `5'GGCGGCGGATCTGCGATACCAAAATTTTATC3`         |
| polhUP3| `5'AAAGGATTATATACGAGATCAATGACG3`             |
| polhDP3| `5'AGAGGATTATATACGAGATCAATGACG3`             |
| polhUP2| `5'AAAGGATTATATACGAGATCAATGACG3`             |
| polhDP2| `5'AGAGGATTATATACGAGATCAATGACG3`             |
| polhUP3| `5'AAAGGATTATATACGAGATCAATGACG3`             |
| polhDP3| `5'AGAGGATTATATACGAGATCAATGACG3`             |
| M13F   | `5'GTTTTCACCAAGCAAG3`                        |
| M13R   | `5'ATGGAATACGATACGATCTGGTATG3`               |
| e25UP4 | `5'GGGCTCTGCTGATGATGCAGT3`                    |

Sequences of restriction sites are underlined.

doi:10.1371/journal.pone.0065635.t001

A fragment containing the polh (nt4355-5257) was amplified with the primers polhUP2 and polhDP2, and inserted into the PsiI and SphI sites of pFastBac1 to obtain pFB-PH-2. Another fragment containing e25 under control of p10 promoter was amplified from pFB-P10-e25 with the primers P10UP3 and e25DP2, and inserted between the XbaI and SacI sites of pFastBac1 to construct pFB-P10-e25-PH. Using the primer pairs P10UP3 and e25DP2, and inserted between the SacI and SnaBI sites of pFastBac1 to make pFB-P10-e25. Another fragment containing polh (nt4300-5257) was amplified with primers polhUP3 and polhDP3 and inserted between the XbaI and PstI sites of pFB-P10-e25, producing pFB-P10-e25-PH.
95°C for 10 s, 60°C for 30 s. The results were analyzed using CFX Manager 2.1 (Bio-Rad) software.

GUS assays

Late gene expression (gus under control of the promoter of AcMNPV gb39) assays were done following a previous protocol with modifications [44]. Sf9 cells seeded in 96-well plates at a density of 1.0×10^5 cells/well were transfected with 0.5 μg of the individual designated bacmids, using liposomes. Grace’s medium was used to make the transfection mixture. Transfected cells were incubated with the transfection mixture at 26°C for 5 h, after which it was replaced with fresh Grace’s medium supplemented with 10% FBS. Cell samples were collected and processed at time points 0, 12, 24, 36, 48 h.p.t., as described below: Medium in with 10% FBS. Cell samples were collected and processed at time incubated with the transfection mixture at 26°C was used to make the transfection mixture. Transfected cells were individual designated bacmids, using liposomes. Grace’s medium warmed (37°C) MUG solution (1 mM in ddH2O) and incubated at 37°C for 10 min. The reaction was terminated by addition of 400 μl of 0.2 M Na2CO3. Fluorescence was then measured with excitation at 365 nm, emission at 455 nm on a FLX800 spectrofluorimeter (Biotek). All GUS expression values were derived from three independent transfections.

Preparation of polyclonal antibodies against E25

A truncated e25 ORF (nt80028-80657), with the 5’-end sequence encoding a putative trans-membrane domain omitted, was amplified as a NcoI -XhoI fragment with primers e25UP4 and e25DP4, and inserted into the correspondent sites of pPROExHTa (Invitrogen) to construct pPRO-e25t, in which the e25DP4, and inserted into the correspondent sites of DE3 pLysS cells; and the HIS-tagged E25t was purified by using Ni-NTA resin (Qiagen), following the manufacturer’s protocol. (DE3) pLysS cells; and the HIS-tagged E25t was purified by using Ni-NTA resin (Qiagen), following the manufacturer’s protocol. The plasmid was transformed into E. coli BL21 (DE3) pLysS cells; and the HIS-tagged E25t protein was purified by using the Ni-NTA resin (Qiagen), following the manufacturer’s protocol. A rabbit was injected with 400 μg of the HIS-tagged E25t protein in complete Freund’s adjuvant. Two weeks after the first inoculation, the animal was boosted with 400 μg of the HIS-tagged E25t protein in complete Freund’s adjuvant. Nine days after the final boost, the animal was bled and the serum was prepared for use in this study.

Western Blot analysis

Sf9 cells seeded in 35 mm plates were transfected with a designated bacmid were harvested at designated time points post transfection. The cell pellets were resuspended individually in 100 μl of PBS and mixed with 25 μl of 5X loading buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 14.4 mM β-mercaptoethanol, 0.1% bromophenol blue), then incubated at 100°C for 5 min. The cell lysate was centrifuged at 12,000 rpm for 5 min.

The protein samples (supernatant) were separated by SDS–12% polyacrylamide gel electrophoresis (PAGE) and transferred to BioTace PVDF membrane (PALL, Life Science) with a liquid transfer apparatuses. The blots were probed with the AcMNPV E25-specific rabbit antiserum prepared above. IRDye-800CW conjugated goat-anti-rabbit antibody (1:10,000) (LI-COR) was used as the secondary antibody. Fluorescence was detected by LI-COR Odyssey. SDS-PAGE and immunohybridizations to western blots were performed in accordance with standard protocols and manufacturer’s instruction [45].

Immuno-fluorescence assays and confocal microscopy

To perform immuno-fluorescence assays, Sf9 cells were seeded on the surface of coverslips placed in 24 mm dishes at 2×10^5 cells/dish and incubated overnight. Then the cells were incubated with infectious supernatant of AcMNPV at a MOI of 5, or, transfected with wt and recombinant bacmids respectively. At 48 h.p.t., or designated time points after infection, the cells on the coverslips were fixed with immunostaining fix solution (Beyotime), incubated with E25-specific antibody, then, incubated with Rhodamine (TRITC)-conjugated goat-anti-rabbit IgG (PTG Lab) (1:60) and stained with Hoechst33258 (Beyotime) sequentially, following standard methods or manufacturer’s recommendation. Finally, the cells were sealed on microscope slides with antifade mounting medium (Beyotime), and subjected to a confocal microscopic assay with a ZEISS LSM710 NLO confocal laser scanning microscope for fluorescence using a wavelength of 488 nm laser line for GFP, 550 nm for Rhodamine, and 352 nm for Hoechst33258. All images were digitally recorded and merged by the use of ZEISS software.

Electron microscopy

For electron microscopy, 1×10^4 Sf9 cells per dish (35 mm) were transfected with 1.0 μg of vAc25koonc, vAc25koonc-P1, vAc25koonc-P10, vAc25koonc-P10, or vAc25koonc-P10. At 72 and 96 h.p.t., cells were fixed, dehydrated, then dislodged with a rubber policeman and precipitated by centrifuge at 3,000 rpm for 3 min. The cell pellets were embedded, sectioned, and stained as described previously [46], then examined with a FEI Tecnai G2 20 TWIN transmission electron microscope at an accelerating voltage of 200 kV.

Acknowledgments

We thank Dr George F Rohrmann for discussions and critical reviewing of the manuscript. Thank Dr Jian-Fang Gui and Jun Zhang for assisting in Confocal microscopy and images. Thank Yuan Hu for proof reading of the manuscript.

Author Contributions

Conceived and designed the experiments: LLL XCL. Performed the experiments: XCL SSW JZ DDQ SMW. Analyzed the data: LLL XCL SSW JZ. Wrote the paper: LLL XCL.

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