Reduction of polyhedrin mRNA and protein expression levels in Sf9 and Hi5 cell lines, but not in Sf21 cells, infected with Autographa californica multiple nucleopolyhedrovirus fp25k mutants

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During cell infection, the fp25k gene of baculoviruses frequently mutates, producing the few polyhedra (FP) per cell phenotype with reduced polyhedrin (polh) expression levels compared with wild-type baculoviruses. Here we report that the fp25k gene of the model baculovirus, Autographa californica multiple nucleopolyhedrovirus (AcMNPV), contains two hypermutable seven-adenine (A7) mononucleotide repeats (MNRs) that were mutated to A8 MNRs and a TTAA site that had host DNA insertions, producing fp25k mutants during Sf21 cell infection. The FP phenotype in Sf9 and Hi5 cells was more pronounced than in Sf21 cells. AcMNPV fp25k mutants produced similar levels of polyhedra or enhanced GFP, which were both under the control of the AcMNPV polh promoter for expression, in Sf21 cells but lower levels in Sf9 and Hi5 cells compared with AcMNPV with an intact fp25k gene. This correlated with the polh mRNA levels detected in each cell line. The majority of Sf21 cells infected with fp25 mutants showed high polh promoter-mediated GFP expression levels. Two cell lines subcloned from Sf21 cells that were infected with fp25k mutants showed different GFP expression levels. Furthermore, a small proportion of Hi5 cells infected with fp25k mutants showed higher production of polyhedra and GFP expression than the rest, and the latter was not correlated with increased m.o.i. Therefore, these data suggest that AcMNPV polh promoter-mediated gene expression activities differ in the three cell lines and are influenced by different cells within the cell line.

INTRODUCTION

Members of the family Baculoviridae are insect-specific viruses with a circular dsDNA genome of 88–180 kbp (Herniou et al., 2012). Interest in baculovirus research is rooted in its potential for biological control of insects and exogenous protein expression, e.g. the development of baculovirus expression vector systems to overexpress proteins in insect cells and larvae for research and pharmaceutical applications (Caron et al., 1990; Medin et al., 1990; Price et al., 1989; Rose et al., 1993; Zheng et al., 2002). The type species of the family Baculoviridae is Autographa californica multiple nucleopolyhedrovirus (AcMNPV), the genetics of which have been studied extensively due to the availability of permissive insect cell lines that support its replication. Its 134 kbp genome contains about 150 genes involved in processes in the viral life cycle, such as viral gene transcription and DNA replication (Ayers et al., 1994).

Early genes are transcribed by the host RNA polymerase and encode enzymes such as viral DNA polymerase for viral DNA replication, and viral RNA polymerase for late gene transcription (Miller & Lu, 1997). Early and late gene expression is separated by viral DNA replication, which is characterized by the biphasic replication cycle to produce budded viruses (BVs), which egress from cells, and occlusion-derived viruses (ODVs), which reside in the nuclei of infected cells (Miller & Lu, 1997). The ODVs are occluded in protein structures called polyhedra, which are mainly composed of the highly expressed 29 kDa polyhedrin protein, a late gene product. Polyhedrin accumulates...
in large amounts in the nuclei of infected cells in the late phase of infection and is required to occlude enveloped nucleocapsids. BVs acquire their envelopes from the plasma membrane when they bud out of cells, whereas the ODV envelope is derived from blebbing of the inner nuclear membrane (INM) (Hong et al., 1997). The blebbing process is facilitated by a 25 kDa ODV envelope protein called FP25K (Rosas-Acosta et al., 2001). FP25K is located in the cytoplasm, at the periphery of the nucleus and in the nuclei of AcMNPV-infected cells (Harrison & Summers, 1995a). It is thought to behave as a chaperone that interacts with viral structure proteins such as E66, E26, gp67 and cellular importin-α-16 to facilitate membrane protein trafficking from the INM of the host cell to envelope newly assembled nucleocapsids in the nucleus before the virions are occluded in the polyhedra (Braunagel et al., 1999, 2004, 2009; Hong et al., 1997).

fp25k mutants accumulate rapidly during serial passage of nucleopolyhedroviruses (NPVs) at a high m.o.i. in insect cells, which often leads to the formation of the polyhedra (FP) per cell phenotype in comparison to the normal many polyhedra (MP) per cell phenotype (de Rezende et al., 2009; Fraser et al., 1995; Kumar & Miller, 1987; Lua et al., 2002). Development of the FP phenotype in insect cells has been reported to be due to mutations in the fp25k gene, caused by host cellular DNA insertions via transposition at the TTAA site in fp25k of certain NPVs (Carstens, 1987; Fraser et al., 1983). The FP phenotype can also be caused by insertions of a single nucleotide in the hypermutable mononucleotide repeat (MNR) sequences, and by base substitution or deletion of nucleotides in fp25k (Beames & Summers, 1988; Bischoff & Slavicek, 1997; Carstens, 1987; Fraser et al., 1983). Single-nucleotide insertions in a stretch of seven-adenine (A7) MNR sequences have been reported in the fp25k gene of Lymantria dispar multiple NPV, Bombyx mori NPV and Helicoverpa armigera NPV (HearSNPV); these insertions are suggested to be due to DNA polymerase slippage during replication of MNR sequences (Bischoff & Slavicek, 1997; Katsuma et al., 1999; Lua et al., 2002). Since the fp25k gene of AcMNPV has two A7 MNRs (Ayres et al., 1994), we hypothesized that the fp25k gene of AcMNPV may also mutate in the two A7 MNRs during viral DNA replication in insect cells.

In addition to the formation of the FP phenotype in cells, inactivation of AcMNPV fp25k also leads to higher titres of BVs in the cell-culture medium, which offers a selective advantage over viruses with an intact fp25k gene (Harrison & Summers, 1995b; Fraser et al., 1995). Mutations in fp25k coincide with the reduction of polyhedrin (polh) mRNA transcription that leads to reduced polyhedrin protein expression in Sf9 cells, also contributing to the formation of the FP phenotype in these cells (Harrison & Summers, 1995a; Jarvis et al., 1992).

In this report, we present evidence that, in addition to transposon insertions, the two A7 MNRs of AcMNPV fp25k were mutated to A8 during passage in Sf21 cells. We also found that the formation of the FP phenotype by AcMNPV fp25k mutants is dependent on different cell lines, and on cells within a cell line that support different levels of polh mRNA and polh promoter-mediated gene expression.

RESULTS

AcMNPV FP viruses contained cellular DNA insertion and MNR replication errors in the fp25k gene

Initial screening for AcMNPV FP mutants was carried out by plaque assay of AcE2P5 BVs generated from Sf21 cells. All viral plaques under agarose overlay were similar and all showed the typical wild-type (wt) AcMNPV large polyhedra plaque phenotype. Fifteen viral plaques were picked; 13 plaques were selected and further purified. Viral DNA was then obtained from BVs, and a pair of fp25k-specific primers amplified two apparent products (1.2 and 1.5 kbp) using AcE2P5 DNA as the template (Fig. 1a). An expected 1.2 kbp fp25k PCR product was amplified from 11 viral plaques with AcP3 and AcP2, and a 1.5 kbp PCR product was produced from two viral plaques with AcP13, as shown in Fig. 1(a). The previously purified recombinant virus AcFP1 (AcGFP) and the parent virus (AcUW1.lacZ; PharMingen) also showed a 1.2 kbp PCR product (Fig. 1a) (Cheng et al., 2001). These PCR products were then directly sequenced.

Sequencing of the PCR products showed that seven viral plaques as shown by AcP3 contained a wt fp25k ORF, each with two A7 MNRs in fp25k, with the first at nt +181–187 (A7-1) and the second at nt +424–430 (A7-2) (Fig. 1c). Four viral plaques as shown by AcP2 had an insertion of an A in the second MNR (A7-2) to form a stretch of eight A residues (A8-2 MNR) that resulted in a frameshift mutation and produced a presumably inactive FP25K protein, with a deletion of the C-terminal 58 aa (Fig. 1b, c). AcFP1 and AcUW1.lacZ (not shown) both had an insertion of an A in the A7-1 MNR, leading to the formation of an A8-1 MNR (Fig. 1b, c). This would result in a putatively inactive FP25K with a deletion of the C-terminal 151 aa (Fig. 1c). PCR product sequencing found that the fp25k gene of AcP13 was interrupted by a 287 bp insert of host-cell DNA at a TTAA site at nt +425, leading to a fp25k fusion with the inserted host DNA (Fig. 1c), which showed 94% sequence identity to a previously reported AcMNPV FP virus, M5, isolated from S. frugiperda cells (Carstens, 1987). AcP13 had a deletion of 73 aa at the C-terminus of FP25K, AcP3, AcP2 and AcP13 were chosen for further study in this report (Fig. 1).

fp25k mutants had a more pronounced FP phenotype in Sf9 and Hi5 cells than in the Sf21 cell line

AcMNPV with or without an intact fp25k gene showed different production levels of polyhedra in Sf21 cells. It was
Some cells with incomplete cytokinesis in the Hi5 cell line were first noticed that AcE2P5 had different production levels of polyhedra in Sf21 cells, suggesting that fp25k gene mutations had occurred during passaging in Sf21 cells [Fig. 2a(i)]. However, when AcP3, AcP2 and AcP13 were used to infect Sf21 cells at an m.o.i. of 10, production of polyhedra was similar for all progeny viruses. AcP3 showed cells with both 5 polyhedra per cell and 1–4 polyhedra per cell and AcP13 and AcP2 also showed production of polyhedra in Sf21 cells similar to that of AcP3 [Fig. 2a(ii–iv)].

Sf9 cells infected with AcP3 showed typical wt production of polyhedra, with the majority of Sf9 cells producing >10 polyhedra per cell [Fig. 2c(i)]. However, for AcP2 infection, about 50% of Sf9 cells showed only a moderately good production of polyhedra (5–10 polyhedra per cell) while the remaining Sf9 cells showed no evidence of production of polyhedra [Fig. 2c(ii)].

Hi5 cells infected with AcP3 also showed typical wt production of polyhedra, with the majority of Hi5 cells showing >30 polyhedra per cell (MP phenotype) [Fig. 2e(i)]. About 95% of Hi5 cells infected with AcP2 had no discernible production of polyhedra in the nuclei and about 5% of infected cells had <8 polyhedra per cell [Fig. 2e(ii)].

Despite the markedly impaired formation of polyhedra in Hi5 cells infected with the fp25k mutants, clusters of Hi5 cells showed a moderately good production of polyhedra (semi-MP phenotype, about 10 polyhedra per cell) in certain areas of the Hi5 cell monolayer [Fig. 2e(iii)]. Polyhedra (3.028 ± 0.807 μm in diameter, mean ± SD, n=65) produced by AcP2 were significantly larger than those produced by AcP3 (1.527 ± 0.471 μm in diameter, mean ± SD, n=67) in Hi5 cells (P<0.01) [Fig. 2e(i, iii)]. Some cells with incomplete cytokinesis in the Hi5 cell clusters showed what appeared to be two nuclei filled with polyhedra [Fig. 2e(iii)]; see Supplementary Video, available in JGV Online]. The presence of these binucleate cells with the semi-MP phenotype suggests that their parental cells are capable of supporting higher production of polyhedra.

**fp25k mutants had reduction in total yield of polyhedra in Sf9 and Hi5 cells, but not in Sf21 cells**

Inactivation of fp25k should result in reduction in yield of polyhedra. We found that the reduction in yield of polyhedra differed among the cell lines tested. There was no yield reduction in the Sf21 cell line infected with AcP2 or AcP13 compared with AcE2P5 and AcP3 (Fig. 2b). In contrast, AcP2 showed significant 3.3- and 4.7-fold reductions in Sf9 and Hi5 cells, respectively, compared with AcP3 (Fig. 2d, f).

**fp25k mutants had a greater reduction of polh promoter-mediated gene expression in Sf9 and Hi5 cells than in Sf21 cells**

As Sf21 cells infected with the fp25k mutants had no reduction in yield of polyhedra, we next asked whether there was any reduction in polh promoter-mediated gene expression of the fp25k mutants in Sf21 cells. AcMNPV bacmid recombinant viruses were generated in which the gfp gene was inserted into either the fp25k locus (AcBacΔ25GFP) or the polh locus (AcBacGFP) for expression. From images taken with equal exposure time at day 3 post-infection (p.i.), it was observed that there were no discernible reductions of GFP expression in most Sf21 cells, but markedly reduced GFP expression was found in some Sf9 cells and most Hi5 cells infected with AcBacΔ25GFP compared with AcBacGFP (Fig. 3a, c, e). When total GFP expression was measured, no significant difference was detected between AcBacGFP and AcBacΔ25GFP in Sf21 cells.
Fig. 2. Comparison of formation of polyhedra by AcMNPV with or without an intact **fp25k** gene between **Sf21, Sf9** and Hi5 cell lines. **Sf21**, Cells were infected with different viruses at an m.o.i. of 10 and photographed at day 3 p.i. (a) Formation of polyhedra by AcMNPV in **Sf21** cells. (i) Phase-contrast microscopy of **Sf21** cells infected with AcE2P5; (ii) AcP3; (iii) AcP13; (iv) AcP2. Arrows point to cells showing the MP phenotype; arrowheads point to cells showing the FP phenotype. (b) Comparison of yields of polyhedra between AcE2P5, AcP3, AcP13 and AcP2 in **Sf21** cells. (c) Formation of polyhedra by AcMNPV in **Sf9** cells. (i) AcP3; (ii) AcP2. (d) Quantitative comparison of yields of polyhedra by AcP3 and AcP2 in **Sf9** cells. (e) Hi5 cells infected with AcP3 or AcP2. (i) AcP3; (ii) AcP2; (iii) AcP2 showing a cluster of six cells with the semi-MP phenotype in Hi5 cells. Arrows point to large polyhedra; arrowheads point to binucleate cells. (f) Quantitative comparison of yields of polyhedra between AcP3 and AcP2 in Hi5 cells. Error bars denote SD; n=3. Treatments with different numbers of asterisks indicate significant differences at $P \leq 0.05$. Bars, 10 μm.
AcMNPV polyhedrin promoter activity in insect cells

**Fig. 3.** Comparison of *polh* promoter-mediated GFP expression of AcMNPV with or without an intact *fp25k* gene between the *Sf*9, *Sf*6b and Hi5 cell lines. Cells of each line were infected with AcBacGFP and AcBacΔ25GFP at an m.o.i. of 10 and photographed in either the phase-contrast or the GFP channel. (a) *Sf*21 cells. (b) Comparison of total GFP expression between AcBacGFP and AcBacΔ25GFP in *Sf*21 cells. (c) *Sf*9 cells. (d) Comparison of total GFP expression between AcBacGFP and AcBacΔ25GFP in *Sf*9 cells. (e) Hi5 cells. Arrowheads point to strongly GFP-expressing Hi5 cells infected with AcBacΔ25GFP. (f) Comparison of total GFP expression (RFU) between AcBacGFP and AcBacΔ25GFP in Hi5 cells. (g) Flow-cytometric analysis of Hi5 cells infected with AcBacΔ25GFP, showing two discrete cell populations. (h) Correlation of GFP-expressing Hi5 cells with increasing AcBacΔ25GFP m.o.i. (i, ii) AcBacGFP; (iii, iv) AcBacΔ25GFP. Error bars denote SD; *n* = 3. Treatments with different numbers of asterisks indicate significant differences at *P* ≤ 0.05. Bars, 10 µm.

(Fig. 3b), whereas GFP expression by AcBacΔ25GFP was reduced by about 50 and 70 % relative to AcBacGFP in *Sf*9 and Hi5 cell lines, respectively (Fig. 3d, f). Despite significant reduction of GFP expression of AcBacΔ25GFP in Hi5 cells, a few cells showed strong GFP expression [Fig. 3e(iv)]. As it was unknown whether these Hi5 cells with strong GFP expression were the result of a higher-dose viral infection, flow cytometry was then used to analyse the ratio changes of Hi5 cells with strong GFP expression when cells were infected with AcBacΔ25GFP at different m.o.i. Two discrete populations of Hi5 cells expressing GFP were detected (Fig. 3g), and an increasing m.o.i. of AcBacΔ25GFP did not increase the ratio of Hi5 cells with strong GFP expression (Fig. 3h).

### *fp25k* gene repair restored the yield of polyhedra in *Sf*9 and Hi5 cell lines

In order to confirm that the single *fp25k* mutation was responsible for the reduction in yield of polyhedra and *polh* promoter-mediated GFP expression in *Sf*9 and Hi5 cells, but not in *Sf*21 cells, an AcMNPV *fp25k* complement virus (AcP3FP25r) was generated. Infection of *Sf*21, *Sf*9 and Hi5 cells with AcP3 all showed wt production of polyhedra, but AcP3A25GFP showed a typical FP phenotype in *Sf*9 and Hi5 cells compared with that in *Sf*21 cells (Fig. 4a, b, d, e, g, h). AcP3FP25r showed similar production of polyhedra to AcP3 in all three cell lines (Fig. 4a, d, g) and showed no difference in yield of polyhedra compared with AcP3 (Fig. 4b, e, h). All of this evidence suggests that the *fp25k* gene of AcMNPV is responsible for the reduction in yield of polyhedra in *Sf*9 and Hi5 cells.

### *fp25k* mutations reduced polyhedrin mRNA levels in *Sf*9 and Hi5 cells, but not in *Sf*21 cells

Reduction of *polh* mRNA levels of AcMNPV *fp25k* mutants compared with wt in *Sf*9 cell infection was reported (Harrison *et al.*, 1996). Whether *polh* mRNA levels of *fp25k* mutants in other insect cell lines are reduced has never been investigated. To test whether *polh* mRNA levels of *fp25k* mutants are reduced in other insect cell lines, *polh* mRNA levels of AcP3A25GFP were compared with those in its parent virus AcP3 and its repair virus AcP3FP25r at 48 h p.i. qRT-PCR of *polh* mRNA showed no difference in *Sf*21 among the three viruses (Fig. 4c), but AcP3A25GFP showed about 50 and 70 % reduction in *Sf*9 and Hi5 cells, respectively (Fig. 4f, i). Similar reductions were also observed for AcP2, AcFP1 and AcP13 in comparison to AcP3 in their infections of *Sf*9 and Hi5 cell lines, but not in the *Sf*21 cell line (Fig. S1).

**Two cloned *Sf*21 cell lines infected with an *fp25k* deletion virus differed in GFP expression**

No difference of *polh* mRNA levels was detected between AcP3 and AcP3A25GFP infections in *Sf*21 cells, (Fig. 4c) but some *Sf*21 cells infected with *fp25k* mutants showed reduced production of polyhedra [Fig. 2a(iii–iv)]. Therefore, we cloned two *Sf*21 cell lines, designated *Sf*6b and *Sf*10s. When *Sf*6b was infected with AcBacΔ25GFP or AcBacGFP at an m.o.i. of 10, no discernible difference in GFP expression was found (Fig. 5a). Measurement of total GFP also confirmed no difference between AcBacΔ25GFP and AcBacGFP infection in *Sf*6b cells (Fig. 5b). In contrast, *Sf*10s cells infected with AcBacA25GFP showed a noticeable reduction in GFP expression (about 50 %) in the majority of cells compared with AcBacGFP (Fig. 5d).

**DISCUSSION**

The *Sf*21 cell line was established from the pupal ovaries of *S. frugiperda* and the *Sf*9 cell line was clonally derived from *Sf*21 cells (Granados *et al.*, 2007; Vaughn *et al.*, 1977). The Hi5 cell line was marketed by Invitrogen after its initial cell line BTI-Tn-5B1-4 was cloned from *T. ni* (Granados *et al.*, 1994). The BTI-Tn-5B1-4 cell line is smaller in cell size, and the Hi5 cell line that had been passaged 360 times showed a reduction in protein expression compared with AcBacΔ25GFP (Fig. 5e). It was also noticed that some *Sf*10s cells infected with AcBacΔ25GFP showed stronger GFP expression than the rest. This might be due to changes in some *Sf*10s cells during passage. These data suggest that there may be differing factors, such as cellular proteins, between the *Sf*10s and *Sf*6b clones that affect the *polh* promoter activities in these cells, and the levels of these factors may change during cell passage.

Earlier experiments on reduction of *polh* expression of AcMNPV *fp25k* mutants were conducted in *Sf*9 cells
(Harrison et al., 1996). Even though the mechanisms behind the reduced polyhedrin production of AcMNPV fp25k mutants in Sf9 cells are not well understood, the unaltered polh expression in Sf21 cells and the cloned Sf6b cell line may suggest that cellular proteins in these cells can regulate polh promoter-mediated gene expression of AcMNPV fp25 mutants, either directly or indirectly.

The Sf21, Sf9 and Hi5 cell lines are all highly permissive for AcMNPV infection. Reduction of polh expression of AcMNPV fp25k mutant viruses in Sf9 and T. ni cells (Tn368) leading to poor production of polyhedra has been well documented (Giri et al., 2010; Harrison et al., 1996; Hink & Vail, 1973). Our results showing reduced polh expression of AcP2 in Sf9 and Hi5 cells (Fig. 2c, d, e, f)
support these early reports (Harrison et al., 1996; Hink & Vail, 1973). This is further supported by the results of AcBac\textsubscript{D25GFP} infection in both Sf\textsubscript{9} and Hi5 cells (Fig. 3c, d, e, f). The reduction of GFP expression by AcBac\textsubscript{D25GFP} in the two cell lines might be due to the positional effects of the polh promoter controlling GFP expression at the \textit{fp25k} locus. However, this hypothesis is not supported by the similar GFP expression of AcBac\textsubscript{D25GFP} in Sf\textsubscript{21} cells (Fig. 3a, b). The finding of no reduction of polyhedra and polh mRNA in the Sf\textsubscript{21} cell line infected with AcP2 is a surprise to us (Figs 2a, b and 4a–c). However, this finding is supported by an earlier report that 19 \textit{lef} genes of AcMNPV without \textit{fp25k} can support high polh promoter-mediated gene expression to the levels of AcMNPV viral DNA transfection in transient expression assays in Sf\textsubscript{21} cells (Rapp et al., 1998). The isolation of a cell line with high GFP expression (Sf\textsubscript{6b}) from Sf\textsubscript{21} cells is in line with the generation of high-protein-expression clones of Hi5: two clonal cell lines from Hi5 cells, H5CL-B and H5CL-F, are able to support higher protein expression than the parental Hi5 cell line (Li et al., 2003). We also observed that the majority of Hi5 cells infected with AcBac\textsubscript{D25GFP} showed weak GFP expression, whereas certain Hi5 cells expressed higher levels of GFP [Fig. 3e(iv)], suggesting that there is a subset of cells in the Hi5 cell line.

Fig. 5. Comparison of polh promoter-mediated GFP expression of AcMNPV with or without an intact \textit{fp25k} gene in clonal Sf\textsubscript{21} cells. Cloned Sf\textsubscript{21} cells (Sf\textsubscript{6b} and Sf\textsubscript{10s}) were infected with AcBacGFP or AcBac\textsubscript{D25GFP} at an m.o.i. of 10 and photographed with a fluorescence microscope system at day 3 p.i. with the same exposure time. (a) GFP expression of Sf\textsubscript{6b} cells infected with AcBacGFP or AcBac\textsubscript{D25GFP}. (b) Quantitative comparison of total GFP expression (RFU) of Sf\textsubscript{6b} cells infected with AcBacGFP or AcBac\textsubscript{D25GFP}. (c) GFP expression of Sf\textsubscript{10s} cells infected with AcBacGFP or AcBac\textsubscript{D25GFP}. (d) Quantitative comparison of total GFP expression (RFU) of Sf\textsubscript{10s} cells infected with AcBacGFP or AcBac\textsubscript{D25GFP}. Error bars denote SD; n=3. Treatments with different numbers of asterisks indicate significant differences at \(P \leq 0.05\). Bars, 10 \(\mu\)m.

Fig. 6. Model explaining different polh promoter activities of AcMNPV \textit{fp25k} mutants and the wt virus in insect cells.
that support high GFP expression driven by the late polh promoter of AcBacΔ25GFP. An early report suggests that higher m.o.i. infection of AcMNPV correlates with higher rates of polyhedrin synthesis and nuclear localization of polyhedrin (Jarvis et al., 1992). Therefore, these strongly GFP-expressing Hi5 cells might be the result of more viral genomes in the cells during infection at high m.o.i. (10), but increasing the m.o.i. (from 5 to 100) of AcBacΔ25GFP did not increase counts of Hi5 cells with high GFP expression as assessed by flow cytometry, suggesting no such correlation between m.o.i. and late polh promoter-mediated GFP expression (Fig. 3g, h). This is consistent with the observation that clusters of MP promoter activity in the cytoplasm is able to interact with cellular importin-a-16 to facilitate viral membrane protein transporting to the INM (Braunagel et al., 2009), FP25K in the nucleus may also interact with viral or cellular nuclear proteins to regulate AcMNPV polh promoter activity. The differences in polh promoter activity in the Sf21 and Hi5 insect cell lines might be due to the presence of viral or cellular factors in these cells that regulate AcMNPV polh promoter activity directly or indirectly. These viral or cellular factors might substitute FP25K to enhance polh promoter activities in some of the insect cells, and could be missing in other insect cells with reduced polh promoter activities. The other plausible explanation for the lower polh promoter activity in some Sf21 and Sf9 cells and in the majority of Hi5 cells might be that these viral or cellular factors serving as repressors bind either to the polh promoter or to one or more LEFs to downregulate polh promoter activity. In the majority of Sf21 cells and in a small population of Hi5 cells, these viral or cellular nuclear protein(s) might be absent or present at very low levels; therefore, the polh promoter activity is not downregulated. The reason that the polh promoter can achieve optimal activity with a functional fp25k gene might be that FP25K located in the nucleus can bind to these cellular or viral nuclear protein(s) to sequester them and prevent them from binding to the LEFs, thereby allowing higher polh gene promoter activity (Fig. 6). This model is currently under testing using different biochemical approaches that will lead to a better understanding of why some Hi5 cells can support high polh promoter-mediated gene expression of AcMNPV fp25k mutants.

**METHODS**

**Identification and isolation of fp25k mutants.** A plaque assay of the amplified AcE2P5 (AcMNPV E2 passaged five times in Sf21 cells) was performed in Sf21 cells following the agarose overlay method (O’Reilly et al., 1992). For each plaque, three rounds of plaque assay were performed to maximize purity (O’Reilly et al., 1992). To verify viral plaque purity and to identify fp25k mutants, viral DNA was extracted from BVs produced from individual viral plaques for PCR analysis with a pair of primers (FP-F and FP-R in Table S1) with an expected amplicon of 1.2 kbp (Ayres et al., 1994). DNAs from each viral plaque, AcE2P5, AcFP1 (AcGFP) and its parental commercial vector AcUW1.lacZ (PharMingen) were used as templates in PCR (Cheng et al., 2001). The amplified DNA products were analysed by agarose gel electrophoresis and purified for sequencing using either the FP-F or FP-R primer to compare with the wt fp25k for insertion and deletion of DNA sequences using Lasergene (DNASTAR).

**Construction of additional FP viruses.** To facilitate comparison of gene expression between AcMNPV with and without a functional fp25k gene in different cell lines and individual cells, another reporter gene, the gfp gene, was cloned into the fp25k locus of AcMNPV using bacmid technology. To generate a bacmid with a functional fp25k to express GFP, the gfp gene was retrieved from pBlueGFP, which had an 88 bp AcMNPV polh promoter to control GFP expression (Cheng et al., 2001), by BamHI and Xhol digestion and cloned between the BamHI and Xhol sites of pFASTBACI to produce PFB-GFP. The PFB-GFP DNA was used to transform DH10Bac Escherichia coli cells (Invitrogen) to generate a bacmid containing gfp at the polh locus (AcBacGFP). To generate a bacmid with the fp25k deletion, a transfer vector was prepared. About 500 bp of homologous recombination sequences up- and downstream of the fp25k gene were amplified by two pairs of primers by PCR using the high fidelity Pfu polymerase (Agilent Technologies). For the upstream fragment, primers 25KPU-HindIII were used (Table S1). For the downstream fragment, a primer set of 25KPD-F-Xhol and 25KPD-R-HindIII was used (Table S1). The amplified DNA fragments were treated with T4 polymerase to add A, to facilitate cloning in pGEM-T Easy (Promega) for sequence confirmation. To assemble a transfer vector, the up- and downstream sequences were retrieved from the clones by digestion with the respective restriction endonucleases. The gfp expression cassette was retrieved from pBlueGFP by EcoRV/Xhol digestion (Cheng et al., 2001). The two homologous recombination DNA fragments and the gfp expression cassette were ligated to the sites between EcoRI and HindIII of pUC19 with T4 DNA ligase (New England Biolabs) for transformation of DH10B cells to produce the transfer vector pUC25kGFP. To insert the gfp expression cassette at the fp25k locus, the bacmid DNA (bMON14272) in DH10Bac E. coli cells (Invitrogen) was extracted. The purified bacmid DNA and transfer vector pUC25kGFP were used to co-transfect Sf21 cells using polyethyleneimine transfection (Oguy et al., 2006). The recombinant bacmid (AcBacΔ25GFP) with an inactivated fp25k was plaque-purified and authenticated by the PCR method (Cheng et al., 2001; O’Reilly et al., 1992).
To confirm the functions of fp25k in gene expression in different cell lines, a repair virus was generated by a two-step method. First, the fp25k gene of AcP3 was inactivated by insertion of the gfp expression cassette using vector transfer pUC25kGFP, as described above. To repair the fp25k of AcP3A25GFP, a 1.5 kbp PCR fragment was amplified with a pair of primers located about 500 bp up- and downstream of the fp25k gene (25kPU-F-EcoRI/25KPD-R-HnaI3 in Table S1) with AcP3 DNA as template, cloned into pGEM-T Easy (Promega) and sequenced to produce the repair transfer vector pGEMT25k. S21 cells were co-transfected with AcP3A25GFP viral DNA and pGEMT25k DNA to generate recombinant fp25k repair virus AcP3FP25r by plaque purification (Cheng et al., 2001; O’Reilly et al., 1992). All viruses were amplified once in S21 cells to generate the viral stocks.

**Comparison of production of polyhedra between viruses in cell culture.** Titres of purified AcMNPV viruses were obtained based on the real-time quantitative (q)PCR method with a pair of primers (pp34F/p34R, Table S1) for the AcMNPV pp34 gene (Lo & Chao, 2004). To compare production of polyhedra, the three cell lines were seeded separately in 35 mm tissue-culture dishes at 10^6 cells per dish. The cells were infected with the plaque-purified AcP3, AcP2, AcP13, AcP3A25GFP and AcP3FP25r viruses at an m.o.i. of 10 from the same viral stocks in triplicate (O’Reilly et al., 1992). At day 3 p.i., infected cells were photographed using a SPOT digital camera attached to a Nikon inverted microscope. The cells in the dishes were counted with a haemocytometer and then lysed with 0.5 % SDS, and the polyhedra were purified by centrifugation at 5000 g for 5 min and then enumerated using a haemocytometer. Differences in yield of polyhedra between AcMNPV with or without a functional fp25k gene in different cell lines were analysed by Student’s t-test in Excel (Microsoft), with a significance level of P<0.05.

**Quantification of GFP expression.** The three insect cell lines were infected with AcBacGFP or AcBacA25GFP at an m.o.i. of 10 as described above and then lysed with 250 μl 0.5 % SDS at day 3 p.i. GFP expression of the total lysate was measured with a Shimadzu RF5301PC spectrophotometer with the associated software (version 1.2). Cell infection was performed in triplicate and the mean GFP yields were from three independent cell infections. Differences in the total amount of GFP between the viruses in different cell lines were analysed by Student’s t-test in Excel (Microsoft).

**Flow-cytometry analysis.** To test whether strongly GFP-expressing Hi5 cells infected with AcBacA25GFP were due to higher virion entry or cell type, Hi5 cells were infected with AcBacA25GFP at increasing m.o.i. (5, 10, 20, 40 and 100) in triplicate. At day 3 p.i., the infected cells were harvested and analysed by flow cytometry using FACScan equipment with the associated software, CellQuest Pro version 5.2 (Becton Dickinson). Percentages of strongly and weakly GFP-expressing cells were analysed using Excel (Microsoft).

**Comparison of polyhedrin mRNA levels.** The three insect cell lines were infected with AcP3, AcP3A25GFP or AcP3FP25r at an m.o.i. of 10 as described above and, at 48 h p.i., cells were harvested and transferred to 1.5 ml Eppendorf tubes followed by a 500 g centrifugation for 5 min. Total RNA from the cell pellets was purified using a TRIzol reagent kit (Invitrogen). The purified total RNA was quantified by spectrometry and 1 μg of each RNA sample was used as template for cDNA synthesis using an oligo dT primer and the reverse primer 28S-R (for input normalization) according to Xue & Cheng (2010) using a DyNAmo cDNA Synthesis kit (New England Biolabs). Quantification of polh mRNA relative to 28S rRNA was analysed by qRT-PCR using SYBR Green Supermix (Bio-Rad) and a Bio-Rad iCycler IQ with a pair of polh primers (Table S1). Copy numbers of polh mRNA relative to 28S rRNA between the viruses with and without an intact fp25k gene in cell infection were calculated (Pfaffl, 2001; Xue & Cheng, 2010; Xue et al., 2010).

**Cloning of S21 cells.** S21 cells were diluted serially in 96-well plates in TNM-FH medium (Invitrogen). Wells containing cells were examined under a microscope to determine the number of cells in each well. Wells with a single cell were marked and incubated until a colony of cells was established. These individual cell clones (passaged seven times) were used for comparison of gene expression between AcMNPV with and without an intact fp25k gene.

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