Removal of transposon target sites from the *Autographa californica* multiple nucleopolyhedrovirus *fp25k* gene delays, but does not prevent, accumulation of the few polyhedra phenotype

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Low-cost, large-scale production of the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) using continuous insect cell culture is seriously hindered by the accumulation of AcMNPV mutants. Specifically, few-polyhedra (FP) mutants, with a reduced yield of occluded virus (polyhedra) and decreased infectivity, usually accumulate upon passaging in cell culture. FP mutations result from transposon insertions in the baculovirus *fp25k* gene, leading to significantly reduced levels of FP25K protein synthesis. This study evaluated the effects of removing the transposon insertion sites from the wild-type baculovirus *fp25k* gene; the mutated virus was denoted Ac-FPm. Specifically, this study involved a detailed comparison of wild-type (WT) AcMNPV and Ac-FPm with regard to the proportion of cells having polyhedra, number of polyhedra per cell, the fraction of empty polyhedra, number of occlusion-derived viruses per polyhedron, number of nucleocapsids in the nuclei, FP25K protein synthesis and genetic analysis of the *fp25k* gene. Removal of TTAA transposon insertion sites from the *fp25k* gene stabilized FP25K protein synthesis and delayed the appearance of the FP phenotype from passage 5 to passage 10. Electron micrographs revealed that more virus particles were found inside the nuclei of cells infected with Ac-FPm than in the nuclei of cells infected with WT AcMNPV (at passage 10). Abnormalities, however, were observed in envelopment of nucleocapsids and virus particle occlusion within Ac-FPm polyhedra. Thus, the FP phenotype appeared in spite of continued FP25K protein synthesis, suggesting that mechanisms other than *fp25k* gene disruption can lead to the FP phenotype.

INTRODUCTION

Baculoviruses offer an environmentally friendly approach to controlling insect pests and have been used successfully for the management of various lepidopteran (moth) pests of crops and forests (Moscardi, 1999; Murhammer, 1996; Szewczyk et al., 2009). A potential market for recombinant baculovirus insecticides has already been established (Black et al., 1997; DuPont, 1996; Smith et al., 2000). Baculoviruses can be produced either in insect larvae or by insect cell culture. Production in insect larvae is low cost but labour intensive unless a robotic system is employed (Gard, 1997). Cell culture production in batch bioreactors becomes cheaper than larval production as the production scale increases (Black et al., 1997; van Lier et al., 1990).

Furthermore, it has been estimated that baculoviruses can be produced in a continuous bioreactor system for 50% of the cost of producing them in batch bioreactors (Rhodes, 1996; Tramper & Vlak, 1986). Unfortunately, baculoviruses are prone to mutant accumulation in continuous cell culture due to repeated passaging. Consistent with this premise, the yield of polyhedra (the form of virus used as the pesticide) and number of viruses per polyhedron decreased significantly by 25 days in a continuous bioreactor system due to few-polyhedra (FP) (Fraser et al., 1983; Harrison & Summers, 1995b) and defective-interfering particle (DIP) mutant accumulation (Kompier et al., 1988; Kool et al., 1991). FP mutations are common in baculoviruses used for the management of lepidopteran pests, including *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV).
(Fraser et al., 1983), Lymnantria dispar MNPV (Bischoff & Slavicek, 1997), Galleria mellonella MNPV (GmMNPV) (Fraser & Hink, 1982), Helicoverpa armigera NPV (HaNPV) and Anticarsia gemmatalis MNPV (AgMNPV) (de Rezende et al., 2009). Hence, overcoming such mutations is an important step in enabling continuous large-scale production of baculovirus biopesticides that will make them more cost competitive with chemical pesticides. The work described here focused on investigating strategies to overcome the FP mutation.

Several studies have demonstrated the connection between serial passage of baculovirus in cell culture and the appearance of FP mutants (Cusack & McCarthy, 1989; Fraser & Hink, 1982; Hink & Strauss, 1976; Knudson & Harrap, 1975; MacKinnon et al., 1974; Potter et al., 1976). FP mutants are characterized by fewer cells containing polyhedra, fewer polyhedra per cell and fewer or no viruses per polyhedron (Harrison & Summers, 1995b). Most of these FP mutants result from the incorporation of host cell DNA into a specific region (HindIII-I fragment of AcMNPV) of the baculovirus genome, thereby disrupting FP25K protein synthesis (Beames & Summers, 1988; Carstens, 1987; Fraser et al., 1983). Examples of such insertions are cell-derived transposable elements such as IFP2 (‘piggyBac’; Cary et al., 1989), TFP3 (‘tagalong’; Wang et al., 1989), hitchiker (Bauser et al., 1996) and TED (Miller & Miller, 1982). There is evidence of host cell insertions in other regions of the AcMNPV genome, e.g. in the 94k and da26 genes, that also lead to the FP phenotype (Friesen & Nissen, 1990; Kumar & Miller, 1987; O’Reilly et al., 1990). FP25K protein synthesis is associated with enhanced biosynthesis and nuclear localization of the polyhedrin protein during the early occlusion phase and acts as a switch from budded virus (BV) production to occlusion-derived virus (ODV) production (Harrison et al., 1996; Jarvis et al., 1992). Furthermore, it has been demonstrated that fp25k gene deletion or an insertion mutation into the fp25k gene leads to the FP phenotype with (i) enhanced BV production (Harrison & Summers, 1995b; Kelly et al., 2008; Wu et al., 2005), (ii) increased synthesis of some BV structural proteins (e.g. GP64, BV E-26 and VP39) (Beniya et al., 1998; Braunagel et al., 1999), (iii) reduced levels of some occluded virus envelope proteins (Harrison et al., 1996), (iv) decreased post-mortem liquefaction of the larval host (Nakanishi et al., 2010), (v) decreased E66 protein synthesis and altered intranuclear transport thereof (Rosas-Acosta et al., 2001), and (vi) production of virions with aberrant morphology (Harrison & Summers, 1995b). Hence, overcoming fp25k gene mutation is a necessary criterion to maintain proper polyhedra and ODV production in continuous cell culture.

Harrison & Summers (1995b) reported that an insertion mutation in the fp25k gene led to the FP phenotype and that normal polyhedron production and the occlusion process could be restored following reinseration of the wild-type (WT) fp25k gene into the virus. Specifically, the FP phenotype primarily results from transposon insertion into TTAA target sites in the fp25k gene (Beames & Summers, 1990; Fraser et al., 1985). Therefore, this study investigated the effect of modifying the 13 TTAA sites in the fp25k gene, without altering the protein’s amino acid sequence, on FP phenotype accumulation.

**RESULTS**

Mutations made in the fp25k gene to remove the TTAA sites (Fig. 1) resulted in a modified AcMNPV, denoted Ac-FPm.

**Characterization of WT AcMNPV and Ac-FPm virus at passage 1**

The initial passage (i.e. passage 1) of WT AcMNPV and Ac-FPm was characterized to provide a basis for assessing changes following passaging in Sf-21 cells. Briefly, the following properties of Sf-21 cells infected with either of these baculoviruses were monitored: (i) cell density and viability, (ii) BV production, (iii) polyhedron production, (iv) FP25K protein synthesis and (v) lethality to insect larvae. Comparing the characteristics of Ac-FPm-infected cells with those of WT AcMNPV-infected cells demonstrated that (i) the time courses of cell density and viability, BV production and FP25K protein production were similar (data not shown) and (ii) there were no significant differences in the distribution of polyhedra per cell (P>0.05, n=3 fields of view). Furthermore, the toxicity of the two baculoviruses against Heliothis virescens (LC50 values) was similar (Table 1). These results indicated that polyhedron production, infectivity, FP25K synthesis and toxicity to insects for WT AcMNPV and Ac-FPm were essentially the same at passage 1.

**Fig. 1.** TTAA sites mutated in Ac-FPm. Circles represent the 13 TTAA sites in the WT AcMNPV fp25k gene that were changed in Ac-FPm. The names of known mutants involving transposon insertion into TTAA sites are indicated. Closed triangles denote the TTAA sites of known insertions of host DNA/transposons into AcMNPV or GmMNPV mutants (Bischoff & Slavicek, 1997). The sites of multiple TTAA sequences in close proximity are indicated by a dashed arrow, with the number in parentheses denoting the number of TTAA repeats. Sites at which the mutation led to reversion of FP25K during passaging are denoted by solid arrows, with the numbers above the arrows denoting the number of mutations (reversions)/total number of isolates investigated.
Cells with polyhedral and the FP phenotype

The polyhedral content and FP phenotype (<10 polyhedral per cell) of the infected cells were monitored by light microscopy. For WT AcMNPV-infected cells at 96 h post-infection (p.i.), the percentage of cells with polyhedra in passage 5 was significantly less ($P < 0.05$) than that of WT passage 1 (Fig. 2a). In contrast, for Ac-FPm-infected cells there was no significant difference between passages 1 and 5 ($P > 0.05$). Although a difference was observed between WT AcMNPV and Ac-FPm infection ($P < 0.05$) in the percentage of polyhedra-producing cells at passage 5, no significant difference was observed by passage 15 (Fig. 2a).

Similarly, a significant increase ($P < 0.05$) in the FP phenotype was noted between passages 1 and 5 of WT AcMNPV-infected cells (Fig. 2b), but no such difference was observed for Ac-FPm-infected cells at 96 h p.i. The FP phenotype for Ac-FPm infection was significantly lower than WT AcMNPV at passage 5, but no significant difference was noted at passage 15 (Fig. 2b).

FP25K protein synthesis

Infected cells isolated at 48 h p.i. and stained with antibodies were examined by immunofluorescence confocal microscopy to identify FP25K and GP64 protein synthesis (GP64 synthesis was used to identify infected cells) (Fig. 3a). The percentage of cells expressing FP25K protein decreased when infected with WT AcMNPV of increasing passage number, i.e. 100, 46.3 and 43.3 % of the infected cell population expressed FP25K protein at passages 1, 6 and 12, respectively. In contrast, no significant change in the percentage of cells containing FP25K protein was observed when infected with Ac-FPm of increasing passage number, i.e. 100, 98.8 and 98.3 % of the infected cell population expressed FP25K protein at passages 1, 6 and 12, respectively (Fig. 3b). These results were confirmed by Western blotting of the proteins from cells infected with the passaged viruses collected at 48 h p.i. (Fig. 3c). Specifically, Western blots of cells infected with passaged WT AcMNPV and Ac-FPm showed a decrease in

Table 1. Dose–mortality response of *H. virescens* neonates infected with WT AcMNPV and Ac-FPm

<table>
<thead>
<tr>
<th>Virus</th>
<th>Slope ± SEM</th>
<th>$LC_{50} \times 10^{-6}$ (95 % CL)$^a$</th>
<th>Potency ratio (95 % CL)$^f$</th>
<th>Heterogeneity ($\chi^2/n$)</th>
<th>$g$ value at 95 % CL$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT AcMNPV P1</td>
<td>1.124 ± 0.151</td>
<td>3.171 (1.646–5.194)$^a$</td>
<td>–</td>
<td>2.316</td>
<td>0.207</td>
</tr>
<tr>
<td>Ac-FPm P1</td>
<td>0.801 ± 0.145</td>
<td>3.414 (1.554–6.111)$^a$</td>
<td>0.841 (0.400–1.711) compared with WT P0</td>
<td>1.648</td>
<td>0.267</td>
</tr>
<tr>
<td>WT AcMNPV P25</td>
<td>0.864 ± 0.118</td>
<td>11.20 (8.066–16.46)$^b$</td>
<td>0.279 (0.147–0.487) compared with WT P0</td>
<td>0.800</td>
<td>0.071</td>
</tr>
<tr>
<td>Ac-FPm P25</td>
<td>1.242 ± 0.127</td>
<td>9.122 (7.195–11.68)$^b$</td>
<td>0.391 (0.236–0.628) compared with Ac-FPm P0</td>
<td>0.550</td>
<td>0.040</td>
</tr>
</tbody>
</table>

$^a$LC$_{50}$ (polyhedra ml$^{-1}$) values with 95 % CL were obtained by running the POLO probit analysis program. For each treatment, LC$_{50}$ values with the same letter were not significantly different if the potency ratio 95 % CL included the value 1.0. For each virus, the values with different superscript letters were significantly different at $P < 0.05$.

$^f$The potency ratio is LC$_{50}$ of the indicated virus/LC$_{50}$ of another virus. If the potency ratio 95 % CL covered the value 1.0, this indicated that both LC$_{50}$ values were the same, i.e. no statistical significance; otherwise they were significantly different (Robertson & Preisler, 1992).

$^d$If $g < 0.5$, then the data fitted the probit model; otherwise, the data did not fit the probit model and the analysis was not valid.

Fig. 2. Comparison of polyhedron production in WT AcMNPV- and Ac-FPm-infected Sf-21 cells after serial passaging at 96 h p.i. (a) Proportion of cells with polyhedra. (b) Fraction of FP phenotype (one to nine polyhedra per cell). The error bars represent the 95 % CL. *, $P < 0.05$ (Student’s $t$-test).
FP25K protein synthesis in cells infected with WT AcMNPV at passage 12. In contrast, significant FP25K synthesis was maintained in Ac-FPm-infected cells at passage 12 as well as at passage 32.

Bioassay

Dose–mortality bioassays with neonates of *H. virescens* demonstrated that the LC50 of both WT AcMNPV and Ac-FPm increased significantly after 25 passages compared with passage 1 (Table 1), indicating a significant reduction in virulence after 25 passages. The LC50 of Ac-FPm from passage 25 was not significantly different from that of WT virus (passage 25) as indicated by the potency ratio (Table 1).

Genetic analysis of the *fp25k* gene

In order to test for transposon insertion during passaging of WT AcMNPV and to evaluate the effect of transposon site removal in Ac-FPm, a detailed sequencing analysis of the *fp25k* gene was performed. PCR amplification of the *fp25k* gene from both WT AcMNPV and Ac-FPm viral DNA (of the heterogeneous population) showed no change in size of the PCR product at passages 12, 15 and 21, indicating that no insertions had occurred in either virus at the TTAA sites (Fig. 4a). Genomic variations within these heterogeneous populations were investigated by conducting sequence analyses on individual clonal isolates. Specifically, 34 isolates from passage 12 were obtained following two rounds of plaque purification (Kelly *et al.*, 2007) of the WT AcMNPV and Ac-FPm viruses. PCR amplification of the *fp25k* gene again demonstrated the absence of insertions in all 34 isolates obtained from WT AcMNPV passage 12 (Fig. 4b). However, evidence of an *fp25k* gene deletion was found in one of the 34 isolates from WT AcMNPV passage 12 (Fig. 4b). Furthermore, DNA sequencing of the *fp25k* gene was performed on 16 randomly selected isolates of the WT and...
Ac-FPm virus clones at passage 12. No evidence of point mutations was found in the WT virus *fp25k* gene, but 3/16 Ac-FPm clones contained point mutations in the modified *fp25k* gene (Table 2), some of which involved reversion of the altered TTAA sites back to TTAA (Table 2, Fig. 1). Similar results were obtained from the analysis of DNA from viral isolates at passage 32 (Table 2). No mutations were detected from sequencing the *fp25k* promoter region (~400 bp upstream of the *fp25k* gene start codon) in any of the WT or Ac-FPm virus isolates from passage 12. As the *da26* gene is another potential part of the AcMNPV genome that may have insertions or deletions to give the FP phenotype (O’Reilly *et al.*, 1990), further PCR analysis of the *da26* gene was performed. No evidence of insertions or deletions was found in the *da26* gene in any of the 34 WT and Ac-FPm virus isolates from passage 12 (data not shown).

**Fig. 4.** (a) PCR amplification of the *fp25k* gene region (1 kb) from passaged WT and Ac-FPm virus (mixed population from passages 12, 15 and 21), indicating no insertion in the *fp25k* gene. (b) PCR amplification of the *fp25k* gene plus *fp25k* promoter region (1.54 kb) from 34 isolates of WT AcMNPV (individual plaques) at passage 12 showing no insertion in the *fp25k* gene; 33/34 of the WT virus isolates retained the complete *fp25k* gene. L, 1 kb size ladder; ML, molecular mass ladder; M, PCR with no DNA; W at passage 1, WT AcMNPV at passage 1; m0, Ac-FPm0; I, isolate; Δ, Δ*fp25k* AcMNPV.
Analysis of polyhedra, ODVs and nucleocapsids outside the polyhedra

Infected cells were analysed at 72 h p.i. by electron microscopy. The presence of no or few polyhedra per cell in WT AcMNPV-infected cells was detected as early as passage 5 (Fig. 5a). Additionally, a representative fraction of the polyhedra had no ODVs and most of the nucleocapsids outside the polyhedra were not enveloped (Figs 5a and 6a). In contrast, at passage 5, cells having many polyhedra (>10 per cell) and polyhedra having many ODVs were predominant in Ac-FPm-infected cells (Figs 5a and 6a) and the number of ODVs per polyhedron was significantly higher than in WT virus-infected cells (Fig. 6b). At passage 10 of the WT AcMNPV, the virus population was dominated by the FP phenotype, with polyhedra having no ODVs (Figs 5a and 6a). By passage 10, a significant fraction of the Ac-FPm virus population had empty polyhedra (Fig. 6a). Although no significant difference was observed in the number of cells having polyhedra and the number of polyhedra per cell between the two viruses at passage 15 (Fig. 2a, b), the number of ODVs per polyhedron was significantly higher in Ac-FPm-infected cells than WT AcMNPV-infected cells at passage 10 [Fig. 6b, analysis of variance (ANOVA), P<0.05, n=12]. At passage 10, the nucleocapsid density in the nuclei (outside the empty polyhedra) was found to be significantly higher in Ac-FPm-infected cells than in WT AcMNPV-infected cells (Student’s t-test, P<0.05, n=15) (Figs 5b and 6c).

DISCUSSION

Effect of virus passaging on FP25K protein synthesis

Although reduced or no FP25K protein synthesis has been correlated with the FP phenotype (Fraser et al., 1983; Beames & Summers, 1988), no previous investigation has evaluated FP25K synthesis in each cell within a cell population infected with passaged virus containing a mixture of FP and MP phenotype. The present immunofluorescence study demonstrated that the fraction of cells with FP25K protein synthesis decreased by more than 50% from passage 1 to passage 6 in WT AcMNPV-infected cells. In contrast, all of the Ac-FPm-infected cells continued to produce FP25K protein at passage 12 (Fig. 3b). Moreover, a Western blot of proteins from passaged Ac-FPm-infected cells revealed that FP25K synthesis was maintained up to passage 32 (>70 days) (Fig. 3c).

Is transposon insertion the only reason for decreased FP25K protein synthesis?

The evidence suggested that the presence of insertions in the fp25k gene is not the only process through which FP25K protein synthesis can be eliminated. For example, 50% of WT (passage 12)-infected cells did not express the FP25K protein (Fig. 3b, c) and 33/34 isolates tested by PCR had a normal-length PCR product (the remaining isolate had a deletion), indicating that there were no insertions in the gene. A potential route through which FP25K protein synthesis can be reduced is by altering the synthesis of viral proteins (e.g. E66) that regulate FP25K synthesis (Bennia et al., 1998). Consistent with these results, Lua et al. (2002) and de Rezende et al. (2009) found that the FP phenotype of HaSNPV and AgMNPV, respectively, resulted from passaging in cell culture without any transposon insertions in the fp25k gene. Neither of these investigations, however, monitored FP25K protein synthesis.

Point mutations in the fp25k gene

The point mutations found in passages 12 and 32 in the fp25k gene are consistent with results of Lua et al. (2002), who found point mutations in the fp25k gene of passaged HaSNPV. Additionally, point mutations of this type could result from DNA replication errors resulting from DNA polymerase slippage (Bischoff & Slavicek, 1997).

The reversion of altered TTAA sites back to the original TTAA sequence (Table 2 and Fig. 1) suggests that some TTAA sites may have an important role in regulating transcription by a cis-acting function, or in regulating

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**Table 2.** Point mutations in the Ac-FPm fp25k gene after passaging

<table>
<thead>
<tr>
<th>Mutants*</th>
<th>No. point mutations</th>
<th>Mutations leading to reversion of TTAA sites</th>
<th>Other mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-FPmP12-m2</td>
<td>1</td>
<td>C24T,</td>
<td></td>
</tr>
<tr>
<td>Ac-FPmP12-m6</td>
<td>3</td>
<td>C24T, G204A, C237A</td>
<td></td>
</tr>
<tr>
<td>Ac-FPmP12-m9</td>
<td>2</td>
<td>C24T, G204A</td>
<td></td>
</tr>
<tr>
<td>Ac-FPmP32-m7</td>
<td>3</td>
<td>C24T, C237T</td>
<td>G214A</td>
</tr>
<tr>
<td>Ac-FPmP32-m20</td>
<td>2</td>
<td>C237T</td>
<td>G214A</td>
</tr>
<tr>
<td>Ac-FPmP32-m21</td>
<td>2</td>
<td>C237T</td>
<td>G214A</td>
</tr>
</tbody>
</table>

*Individual virus isolates were plaque purified from passages 12 and 32 of Ac-FPm. Mutations are indicated.
**Fig. 5.** Electron micrographs of WT AcMNPV- and Ac-FPm-infected Sf-21 cells at passages 5 (P5) and 10 (P10) at 72 h p.i. (a) The FP and MP characteristics showed that formation of the FP phenotype is delayed in Ac-FPm compared with WT AcMNPV. (b) The number of nucleocapsids per area outside the empty polyhedra was greater in Ac-FPm-infected cells than in WT AcMNPV-infected cells at passage 10.
mRNA stability, transport and translation. Similar cis-acting functions have been observed with sequences within the AcMNPV genome (but not in the fp25k gene), e.g. a cis-acting element within the 5’ non-coding region of the ie-1 gene (Pullen & Friesen, 1995) and a cis-acting element upstream of the polh promoter (Kumar et al., 2009).

**FP phenotype in a heterogeneous infected-cell population**

Although many investigations have been performed involving the genetic and electron microscopic analysis of FP clones isolated via plaque purification (Bischoff & Slavicek, 1997; Fraser & Hink, 1982; Harrison & Summers, 1995b; Lua & Reid, 2003; Lua et al., 2002), only a few studies have been conducted to characterize the heterogeneous virus population by electron microscopy (de Rezende et al., 2009; MacKinnon et al., 1974). In this study, the heterogeneous virus population was characterized for passages 5 and 10 with respect to the number of polyhedra-producing cells, the number of polyhedra per cell, the number of ODVs per polyhedron and the number of nucleocapsids outside empty polyhedra (Figs 5 and 6). These results demonstrated that passaging the baculoviruses led to large variations in these parameters compared with the relative homogeneity at passage 1.

**Removal of transposon insertion sites delays the emergence of FP characteristics**

Comparison of WT AcMNPV- and Ac-FPm-infected cells demonstrated that FP phenotype accumulation was delayed in Ac-FPm-infected cells. An unexpected result of this study was a decrease in the percentage of WT AcMNPV-infected cells having polyhedra from passage 1 to passage 5, followed by an increase from passage 5 to passage 15 (Fig. 2a). One possible reason could be a fluctuation in the number of DIPs (mutant virus in which 40–45% of the genome is deleted) in the virus population at different times of passage. Cyclic peaks in DIP formation...
are common during passaging of animal viruses (Dimmock & Marriott, 2006), and DIP genomes could lack the fp25k gene that would lead to the FP phenotype. Consistent with this possibility, gel fractionation of viral genomes from WT-virus infected cells from passage 5 showed that most of the genomes were shorter than those found at passage 1 (data not shown). The fraction of cells containing polyhedra and the number of polyhedra per cell for Ac-FPm- and AcMNPV-infected cells were comparable by passage 15 (Fig. 2) and the fraction of polyhedra containing viruses were comparable by passage 10 (Fig. 6a). These phenomena occurred in spite of the fact that FP25K protein synthesis in Ac-FPm-infected cells (but not in WT AcMNPV-infected cells) was maintained. This suggested that mutations in genes other than fp25k are involved in FP accumulation. Indeed, mutations in other genes, e.g. da26 and 94k, are known to disrupt occlusion body production (Friesen & Nissen, 1990; O’Reilly et al., 1990). In the present study, it was demonstrated that the da26 gene in WT AcMNPV did not contain insertions at passage 12, but the presence of other types of mutation was not investigated. The FP25K protein may interact with other viral proteins to regulate the viral occlusion process, and mutations in any of the genes involved in this process may lead to the FP phenotype. Also, point mutations in the polyhedrin gene can lead to abnormal polyhedron formation and altered polyhedrin protein localization (Bravo-Patiño & Ibarra, 2000; Katsuma et al., 1999; Lin et al, 2000; Nakazawa et al., 1996).

Whilst the fraction of polyhedra containing ODV in Ac-FPm- and AcMNPV-infected cells was comparable at passage 10, the Ac-FPm-infected cells contained more virus both inside and outside the polyhedra (Figs 5b and 6). These observations support the premise that the FP25K protein is involved in targeting the virions to the nucleus. However, the finding that nucleation of the polyhedrin protein around ODVs was greatly diminished in Ac-FPm-infected cells, even in the presence of significant FP25K protein synthesis, suggests that other proteins are involved in this process.

Conclusion

This investigation demonstrated that removal of TTAA sites from the fp25k gene can maintain FP25K protein synthesis for at least 32 passages in cell culture and can delay, but not stop, FP mutant accumulation. Therefore, further studies need to be conducted to determine the strategy necessary to develop a modified AcMNPV conducive to a continuous process for producing baculovirus biopesticides. This will require a comprehensive understanding of the correlation between FP phenotype and mutations in baculovirus genes. Future work may involve a detailed study of mutation patterns during serial passaging by utilizing microarray analysis (Yamagishi et al., 2003). It would also be interesting to determine the viral proteins that interact with FP25K and are involved in FP phenotype formation. Functional studies of proteins (e.g. E26, E25, ODV-E66, GP64, VP39, 94k and p35) may answer questions concerning the mechanism behind the formation of polyhedral crystals, transport of nucleocapsids and polyhedrin nucleation around ODVs. Finally, viruses with modified TTAA sites in the fp25k gene may lead to a more genetically stable baculovirus expression vector system for recombinant protein production in which many virus passages are required to go from a small virus stock to a large-scale batch bioreactor.

METHODS

Cells and virus. The Spodoptera frugiperda IPLB-Sf-21AE cell line (Sf-21) (Vaughn et al., 1977) and AcMNPV strain E2 (Ayres et al., 1994) were used. Sf-21 cells were maintained in 125 ml Erlenmeyer flasks (20 ml working volume) on an orbital shaker at 140 r.p.m. and 27 °C in SF-900 II serum-free medium (Invitrogen). Cell density and viability were monitored with a Vi-Cell (Beckman Coulter) and BV was quantified using an end-point dilution assay as described by O’Reilly et al. (1992). The virus Δfp25K, with the fp25k gene deleted, was used as a control virus (Rosas-Acosta et al., 2001).

Construction of Ac-FPm. The recombinant baculovirus denoted Ac-FPm was constructed by modifying the TTAA transposon target sites in the AcMNPV fp25k gene. Briefly, plasmid pAcE2HindIII-I containing the HindIII-I fragment of AcMNPV E2 was constructed by ligating the HindIII-I fragment into the HindIII site of pUC18. The coding sequence of fp25k in pAcE2HindIII-I was mutated using template-directed ligation and PCR to alter the 13 potential TTAA transposon target sites without changing the amino acid sequence of the FP25K protein. The 11 TTAA sequences within the coding sequence were altered by changing either the second T or first A without changing the resulting amino acid. Nine different primers were constructed with nucleotide changes incorporated for the TTAA sequences. TWO of these primers were used to alter multiple TTAA sequences that occur in close proximity to each other and the other seven primers were used to alter the other seven TTAA sites (Fig. 1). The resulting modified fp25k gene was introduced into the viral genome by co-transfecting Sf-21 cells with the plasmid DNA and AcFPgal DNA. The AcFPgal baculovirus contains a µ-galactosidase gene fused in frame after nt 373 of the fp25k ORF (Harrison & Summers, 1995b). Following a double homologous recombination event, the baculovirus Ac-FPm was produced. Recombinant baculovirus clones were identified by conducting plaque assays and staining for µ-galactosidase activity (the desired clones did not contain this activity). The selected viruses were purified by plaque assay and amplified, and the DNA was extracted to confirm the expected genetic structure by restriction enzyme analysis (O’Reilly et al., 1990). The sequence of the mutated fp25k gene was verified and FP25K protein synthesis was examined by Western blotting. A polyclonal antibody against the FP25K protein was obtained by preparing a fusion protein of FP25K and maltose-binding protein as described by Harrison & Summers (1995a) and then injecting into a rabbit (Elmira Biologicals).

Serial passaging of AcMNPV in vitro. A serial passaging experiment was designed to simulate the baculovirus passaging that occurs in a continuous bioreactor system. For all passages, a 20 ml working volume of Sf-21 cells (in SF-900 II SFM medium supplemented with 10% FBS; Gibco) at a concentration of 0.8 × 10⁶ cells ml⁻¹ was infected with the corresponding baculovirus. Initially, cells were infected at an m.o.i. of 10 with WT AcMNPV or Ac-FPm. Supernatant containing BV was collected for subsequent
passages when the cell viability decreased to 65–70% (at 2–3 days p.i.). This was continued for all passages, with 1.5 ml cell supernatant containing BV being used to infect a fresh cell suspension. All baculovirus stocks were obtained after removing cells by centrifugation at 800 g for 10 min.

Polyhedra quantification. SF-21 cells were infected with passaged WT AcMNPV or Ac-FPm at an m.o.i. of 10. Cells (at least 100) harvested at 96 h p.i. were examined by light microscopy for the presence or absence of polyhedra and the number of polyhedra per cell to determine the distribution of the FP phenotype (defined as <10 polyhedra per cell) and MP phenotype (defined as ≥10 polyhedra per cell). Triplicate flask of infected cells were used for every passage. Cells infected with WT AcMNPV and Ac-FPm were compared using Student’s t-test (Shoemaker et al., 1974) with regard to the fraction of cells with polyhedra and the FP phenotype fraction.

Western blot analysis of FP25K synthesis. At 48 h p.i. for selected passages, infected cell pellets (3 × 10⁶ cells) were collected and lysed by sonication (30 s). Total cellular protein was quantified by the Bradford method using a BSA standard. Proteins in the cell pellet were separated by 12% SDS-PAGE (Bio-Rad) at 180 V with each lane loaded with 25 μg protein. The proteins were blotted onto a nitrocellulose membrane (Bio-Rad) using a TransBlot Semi-Dry Transfer Cell (Bio-Rad) and analysed with a rabbit polyclonal antibody against the AcMNPV FP25K protein at a dilution of 1:100 000 (Sambrook et al., 1989). Bound antibody was detected using goat anti-rabbit IgG conjugated with horseradish peroxidase at a 1:100 000 dilution (Pierce). A SuperSignal West Pico chemiluminescent Substrate kit (Pierce) was used to develop the protein bands on the blot and the protein was quantified using ImageJ software version 1.3.

PCR. The primers fppromoter120up (5'–GGGTTTACGCTGCTCGCGGGCGGC–3'; forward) and Acfp25k165down (5'–CTCTATAGGAAAGG–3'; reverse) were used to amplify the approximately 1.54 kb fp25k gene region (fp25k gene plus fp25k promoter) by PCR as described by Liu et al. (2002). The PCR fragments were sequenced using the primers fppromoter120up, Acfp25k130up (5’–GGGTATATGAGGTCAAACTC–3' and Acfp25k165down. Another set of primers, da26200up (5’–CAACAGCTGCCAATGTACCG–3'; forward) and da26200down (5’–CTGAATATAAGCGCT–3'; reverse), were used to amplify the da26 gene region to check for transposon insertion (O'Reilly et al., 1990).

Immunofluorescence assay. SF-21 cells (0.5 × 10⁶) were infected at an m.o.i. of 20 and incubated at 27 °C, and an immunofluorescence assay was performed as described by Bjerke & Roller (2006) at 48 h p.i. The primary antibody was a mixture of anti-FP25K (1:1000) and anti-GP64 (1:7000) (Novagen) and the secondary antibody was a mixture of Alexa Fluor 568 goat anti-rabbit (1:1000) and Alexa Fluor 488 goat anti-mouse (1:1000) (Invitrogen). The images were photographed with a Bio-Rad MRC-1024 confocal microscope. For each treatment, three fields of view were observed, with each field containing 60–80 cells. FP25K protein synthesis was quantified by calculating the percentage of infected (GP64-positive) cells producing FP25K protein (FP25K positive) per treatment. The percentage of cells infected with WT AcMNPV or Ac-FPm that produced FP25K protein was determined by Student’s t-test (Shoemaker et al., 1974) with a 95% confidence limit (CL) and n=3 (number of fields photographed per treatment).

Bioassay. Lethal-concentration bioassays were performed using the droplet feeding method (Hughes & Wood, 1981). Polyhedra of WT AcMNPV and Ac-FPm (passages 0 and 25) were assayed against neonate Heliothis virescens larvae as described previously with three replicates of 30 larvae per virus concentration (Harrison & Bonning, 2001). The concentrations used were 1.0 × 10⁶, 3.0 × 10⁶, 9.0 × 10⁶, 18 × 10⁶ and 36 × 10⁶ polyhedra ml⁻¹. Larval mortality was scored after mock-infected larvae had pupated. LC₅₀ values were calculated by POLO probit analysis and compared by standard lethal concentration ratio comparison (Robertson & Preisler, 1992).

Transmission electron microscopy. Infected SF-21 cells from passage 5 and 10 at 72 h p.i. were fixed, embedded in resin and sectioned as described by Harrison & Summers (1993b). Sections (100 nm) were stained with 5% uranyl acetate and lead citrate. Samples were examined using a JEOL 1230 transmission electron microscope at 120 kV. The images were used to determine the number of cells with polyhedra, the number of ODVs per polyhedra and the number of nucleocapsids outside the empty polyhedra. Statistical analysis of data was performed using ANOVA in MATLAB with 95% CL.

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REFERENCES


Modified AcMNPV fp25k gene delays FP phenotype


