

Functional analysis of FP25K of *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus

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The *fp25k* gene of *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus (HearNPV) was studied. HearNPV *fp25k* gene transcription was found starting from about 18 h post-infection, and protein could be detected from the same time with antiserum against FP25K. To study the function of HearNPV *fp25k*, a recombinant HearNPV (HaBacWD11) with an enhanced green fluorescent protein (GFP) gene replacing the *fp25k* was constructed using HaBacHZ8, a bacmid of HearNPV that lacks the polyhedrin gene. Growth curve analysis showed that HaBacWD11 produced higher titres of budded viruses (BVs) than its wild-type counterpart HaBacHZ8–GFP. Electron microscopic analysis indicated that at the late stage of infection, the number of intranuclear enveloped nucleocapsids in HaBacWD11-infected cells was much less than that of HaBacHZ8–GFP. A rescue recombinant virus HaBacWD14 was constructed by reintroducing *fp25k* gene into HaBacWD11. The growth curve and electron microscopic analysis of the rescued recombinant confirmed that the increase of BV yield and the decrease of the virion production in infected cells were the result of *fp25k* deletion. The expression of membrane fusion protein (Ha133) and ODV-E66 were studied using the FP25K mutants HaBacWD11 and HaBacHZ8–GFP. Unlike FP25K mutants in *Autographa californica* multicapsid NPV (AcMNPV), which caused an increase in the expression of membrane fusion protein GP64 and a decrease of ODV-E66, no obvious changes at the expression level of Ha133 and ODV-E66 were observed in HearNPV FP25K mutant.

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INTRODUCTION

Serial passaging of baculoviruses in cell culture leads to the few polyhedra (FP) phenotype (Fraser *et al.*, 1983). The most common properties of an FP phenotype are a decrease in the number of polyhedra produced per cell, an increase yield of budded virus (BV), and few or no virions occluded within polyhedra in cells infected with an FP genotype. FP mutants have been reported in several baculoviruses, including *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) (Hink & Vail, 1973), *Trichoplusia ni* MNPV (TnMNPV) (Potter *et al.*, 1976), *Galleria mellonella* MNPV (GmMNPV) (Fraser & Hink, 1982), *Lymantria dispar* MNPV (LdMNPV) (Slavicek *et al.*, 1992), *Orgyia pseudotsugata* MNPV (OpMNPV) (Russell & Rohrmann, 1993) and *Helicoverpa armigera* single nucleocapsid NPV (HearNPV) (Chakraborty & Reid, 1999). The *fp25k* gene was identified as being responsible for the FP phenotype (Fraser *et al.*, 1983; Bischoff & Slavicek, 1997). FP mutation

also affected the post-mortem liquefaction of larval host (Katsuma *et al.*, 1999).

Many proteins seem to be affected by FP25K mutations at the molecular level. It has been reported that mutations in FP25K result in (i) a decreased amount of polyhedrin mRNA and altered transport of polyhedrin protein into the nucleus (Harrison *et al.*, 1996), (ii) significantly decreased amounts of ODV-E66 and impaired transportation of ODV-E66 into the nucleus (Braunagel *et al.*, 1999, 2004; Rosas-Acosta *et al.*, 2001), and (iii) a significant increase in the synthesis of some structural viral proteins of BVs such as membrane fusion protein GP64, BV/ODV-E26 and VP39 (Braunagel *et al.*, 1999). Although the effects produced by FP25K mutants are known, the function of this protein is still unknown. As most of the molecular effects of FP25K were generated from AcMNPV, it remains unclear whether the same effects exist in other baculoviruses.

The HearNPV FP mutant was first reported by Chakraborty & Reid (1999). Lua *et al.* (2002) reported the rapid accumulation of FP mutants in HearNPV. During passage of wild-type HearNPV on HzAM1 cells, the FP phenomenon was observed by passage 6 in all the infected cells. Electron microscopy studies revealed that very few polyhedra were produced in cells that were infected with the FP mutant and in most cases the polyhedra did not contain virions (Lua *et al.*, 2002). The *fp25k* genes of these mutants contained point mutations, insertions or deletions (Lua *et al.*, 2002).

In this work, we first analysed the transcription and expression of *fp25k* in HearNPV-infected cells. We then attempted to study the function of the *fp25k* gene by replacing it with a gene encoding an enhanced green fluorescent protein (GFP) in HaBacHZ8, an infectious polyhedrin null HearNPV bacmid (Wang *et al.*, 2003). The production of BV and intranuclear enveloped nucleocapsid (equivalent to ODV) of the recombinant virus was compared with a control virus HaBacHZ8-GFP, which contained the native *fp25k* gene and an inserted enhanced *gfp* gene. The *fp25k* gene was reintroduced into the recombinant virus to ascertain that no other function was affected by the deletion. In addition, we analysed the expression of BV membrane fusion protein (Ha133) and ODV-E66 in the *fp25k* null mutant. Our results indicated that the deletion of *fp25k* caused a higher BV yield and a decrease in progeny ODVs. Unlike AcMNPV, in which the deletion of *fp25k* would result in an increase of the BV membrane fusion protein GP64 and a decrease of ODV-E66 (Braunagel *et al.*, 1999; Rosas-Acosta *et al.*, 2001), the expression of the membrane fusion protein and ODV-E66 in HearNPV were not significantly affected when *fp25k* was deleted.

METHODS

Insect cells, virus and infection. The cell line HzAM1, originally isolated from *Helicoverpa zea* pupal ovarian tissue (McIntosh & Ignoffo, 1983), was maintained in Grace's insect medium supplemented with 10% fetal bovine serum (Gibco-BRL) at 28 °C and was used for expression analysis. Wild-type HearNPV was originally isolated from diseased *H. armigera* larvae in Hubei province of China (Zhang *et al.*, 1981). The HearNPV-G4 strain is an *in vivo* clone from the wild-type isolate (Sun *et al.*, 1998) and the complete genome sequence of the clone has been reported previously (Chen *et al.*, 2001). HzAM1 cells were infected with haemolymph of HearNPV-G4-infected larvae, the supernatant was collected as primary BV stock and kept at 4 °C. HaBacHZ8 was an infectious bacmid of HearNPV (Wang *et al.*, 2003). Titres of BV were determined by the end-point dilution method (O'Reilly *et al.*, 1992).

Transcriptional analysis of HearNPV *fp25k* gene. HzAM1 cells (3×10^5) were infected with HearNPV-G4 at an m.o.i. of 5. At appropriate time points post-infection (p.i.), the infected cells were collected and the total RNAs were extracted by RNA-solv reagent (Omega). 3'RACE experiment was performed using 3'-full RACE core set (TaKaRa) according to the manufacturer's instructions. Total RNA (1 µg) from each time point was transcribed by AMV reverse transcriptase and an oligo(dT) three-site adaptor primer to synthesize the first strand cDNA. The PCR reaction was followed using the cDNA as template; the primers were a three-site adaptor primer and an *fp25k* gene-specific forward primer with sequence of 5'-ACTCGTGACGCCCTATTACCG-3' (424–445 nt in the *fp25k*

gene). PCR products were analysed by agarose gel electrophoresis. The PCR product derived from the 48 h p.i. sample was purified and cloned into pGEM-T easy vector (Promega) and then sequenced with M13 primers to determine the 3' end of *fp25k* gene transcript.

Expressional analysis of HearNPV *fp25k*. HzAM1 cells (3×10^5) were infected with HearNPV-G4 at an m.o.i. of 5. At appropriate time points p.i., the infected cells were collected and rinsed with PBS. Protein samples were separated on 10% SDS-PAGE and transferred onto nitrocellulose membrane using Semi-Dry Transfer Cell (Bio-Rad) as recommended by the manufacturer. The primary antibody was a rabbit polyclonal antibody against HearNPV FP25K (Wu *et al.*, 2004). The secondary antibody was goat anti-rabbit IgG conjugated with alkaline phosphatase. NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) detection was performed as described (Sambrook *et al.*, 1989).

Construction of *fp25k*-deleted HearNPV bacmid. A pair of primers was designed to amplify the coding sequence of HearNPV *fp25k*, forward primer fp-F with an *EcoRI* site (underlined): 5'-GGCGAATTCAAATGGAACTGATCTAATTAATG-3' (the italic nucleotides are complementary to 44017–44041 nt in HearNPV G4 genome) and reverse primer fp-R with a *NotI* site (underlined): 5'-GGCGCGGCCGCTATGACGGGCAAGGGGGCG-3' (the italic nucleotides corresponded to 43355–43373 nt in HearNPV G4 genome). The PCR fragment was inserted into pGEM-T-easy vector (Promega). Based on this recombinant pGEM-T-easy vector, a 480 bp coding sequence of *fp25k* digested by *HpaI* (43490–43969 nt in HearNPV-G4 genome) was replaced with a gene cassette with a chloramphenicol resistance (*Cm^r*) gene and an enhanced GFP gene under the *hsp70* promoter. This recombinant plasmid was named pFPdel. The 2.4 kb PCR fragment amplified from pFPdel using primers fp-F and fp-R was transformed into *Escherichia coli* BW25113 containing HaBacHZ8 DNA. The *fp25k*-deleted bacmid was generated by homologous recombination in *E. coli*, and was screened by kanamycin and chloramphenicol resistance as described previously (Hou *et al.*, 2002). The positive recombinant was named HaBacWD11. For a control, an enhanced GFP gene was introduced to HaBacHZ8 by using the HearNPV bac-to-bac system (Wang *et al.*, 2003) and this generated the bacmid HaBacHZ8-GFP. The constructs of the recombinant HaBacWD11 and HaBacHZ8-GFP are shown in Fig. 1.

Construction of *fp25k*-rescued bacmid. A pair of primers was designed to amplify the complete sequence of *fp25k* of HearNPV containing its putative promoter and poly(A) signal sequences. Ha-fp6 is the forward primer with an *NcoI* site (underlined): 5'-GGCCATGGCGTGTGGTGGACGAATTTTGGCCAT-3' (the italic nucleotides are complementary to 44214–44236 nt in HearNPV-G4 genome). Ha-fp7 is the reverse primer, with a *KpnI* site (underlined): 5'-GGGGTACCATGGTGACCTATAACGTTTACGA-3' (the italic nucleotides correspond to 43231–43253 nt in HearNPV-G4 genome). The PCR product was cloned into a pGEM-T easy vector. The transfer vector pFastBacDual (Invitrogen Life Technology) was digested with *SmaI* and *BamHI* to knock out the *polyhedrin* and *p10* promoters, and named pFastWD13. The completed *fp25k* fragment from the pGEM-T easy vector was subcloned into pFastWD13 and named pFastWD14. Transposition was performed on the Tn7 attachment site of HaBacWD11 using pFastWD14 as the donor plasmid (Hou *et al.*, 2002). The resulting bacmid was named HaBacWD14. For a control, pFastWD13 was used as the donor plasmid and transposed to HaBacWD11, generating HaBacWD13 (Fig. 1).

All bacmids, identified by PCR and restriction enzyme analysis, were transfected into HzAM1 cells according to O'Reilly *et al.* (1992) and the supernatants of transfection were used as primary stocks of BV.

Detection of *fp25k* expression of the recombinant viruses. HzAM1 cells were infected with HearNPV-G4, HaBacHZ8-GFP,

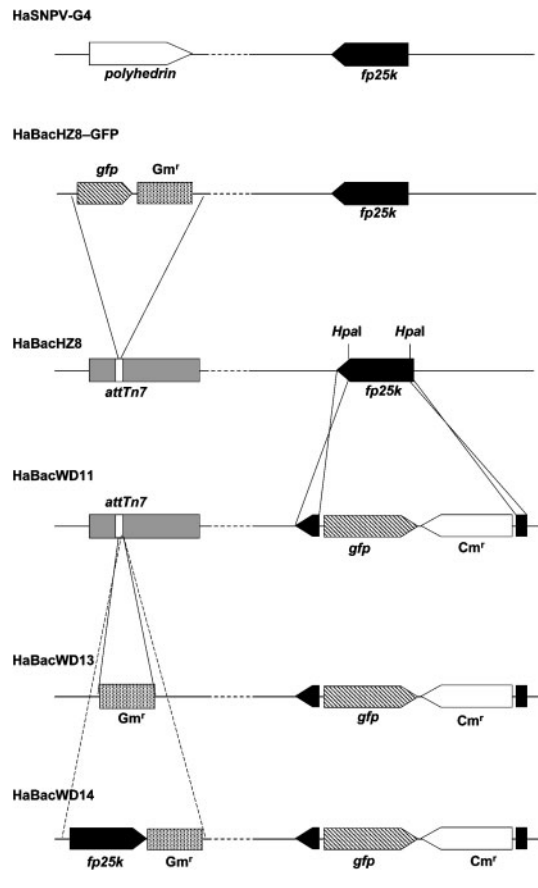


Fig. 1. Schematic representations of HearNPV-G4 and recombinant bacmids. HearNPV-G4 is the wild-type virus and HaBacHZ8 is a HearNPV bacmid. HaBacWD11 is *fp25k*-deleted HaBacHZ8, in which a 480 bp *HpaI* fragment of *fp25k* coding region was replaced by two gene cassettes: *Cm^r* and *gfp*. HaBacWD13 and HaBacWD14 were transposed from HaBacWD11 using the donor plasmids pFastWD13 and pFastWD14. HaBacHZ8-GFP was a control virus containing enhanced GFP as the marker.

HaBacWD11, HaBacWD13 and HaBacWD14. The cells were collected at 48 h p.i., and cellular proteins were separated by 10% SDS-PAGE. Immunoblotting was performed as described above.

Comparison of BV growth curves between recombinant viruses. HzAM1 cells were infected with HaBacHZ8-GFP, HaBacWD11, HaBacWD13 or HaBacWD14 at an m.o.i. of 5. At the appropriate time points p.i., supernatants were collected and the titres were detected using the end-point dilution method. The green fluorescence was used as the infectious marker during the assay. Each virus infection was repeated three times and the growth curves were generated by arithmetic mean data of three infections. The titre values of the four viruses were compared with one-way ANOVA (SPSS).

Electron microscopic observations of cells infected with the recombinant viruses. HzAM1 cells were infected with HaBacHZ8-GFP, HaBacWD11, HaBacWD13 or HaBacWD14 at an m.o.i. of 5. Infected cells were fixed at 96 h p.i. and processed for

electron microscopic analysis. The arithmetic mean numbers of ODV particles were calculated from 20 to 25 cells that contained ODV particles.

Expression of Ha133 and ODV-E66. HzAM1 cells were infected with HaBacHZ8-GFP and HaBacWD11 at an m.o.i. of 5. At the appropriate time points p.i., infected cells were collected and rinsed with PBS. SDS-PAGE and immunoblotting were performed as described above. Anti-HaF1, a polyclonal rabbit antiserum against the N-terminal fragment of Ha133 (F1), was used as the primary antibody to detect Ha133, and a polyclonal rabbit antiserum anti-HaE66 was used for detecting ODV-E66.

RESULTS

Transcription and expression analysis of HearNPV *fp25k*

The coding sequence of HearNPV *fp25k* was located at 43385–44038 bp in HearNPV-G4 genome in the opposite orientation to the *polyhedrin* gene (Chen *et al.*, 2001). The coding region was 654 nt long with a predicted peptide of 217 aa and a molecular mass of 25.4 kDa. At 85 nt upstream of the ATG codon, there is a late baculovirus gene transcription motif, ATAAG. According to the 3'RACE results, the transcriptional product of *fp25k* gene of HearNPV could be detected from 18 to 96 h p.i. (Fig. 2a), which is in agreement with it being a late gene. The sequencing result of the 3'RACE indicated that a poly(A) sequence was added at 110 bases downstream of the translation stop codon. There are two AATAAA motifs located at 65 and 95 nt downstream of the stop codon. Our results suggest that the second AATAAA was used as the poly(A) signal for HearNPV *fp25k*.

Western blot analysis showed that a 26 kDa specific band was detected by an antibody against FP25K from 18 to 96 h p.i. A minor band of 23 kDa was also detectable from 24 to 96 h p.i. After 48 h p.i., a band with a molecular mass of 24.5 kDa also appeared (Fig. 2b). Western blots confirmed that *fp25k* is a late gene.

Construction of recombinant viruses

Four recombinant viruses, HaBacWD11, HaBacHZ8-GFP, HaBacWD13 and HaBacWD14 were constructed according to Methods. Their structures are shown in Fig. 1. All these recombinants were authenticated by PCR and restriction enzymes analysis. The recombinants were infective to HzAM1 cells where green fluorescence was evident (data not shown). The expression of FP25K in the cells infected with these viruses was detected by Western blots. As shown in Fig. 2(c), two forms of FP25K (26 and 23 kDa) were detected at 48 h p.i. in the cells infected with HearNPV-G4, HaBacHZ8-GFP and HaBacWD14, but not in those infected with HaBacWD11 and HaBacWD13. Our results indicate that the recombinants were constructed correctly.

BV growth curves of recombinant viruses

HzAM1 cells were infected with HaBacHZ8-GFP, HaBacWD11, HaBacWD13 or HaBacWD14. The one-step

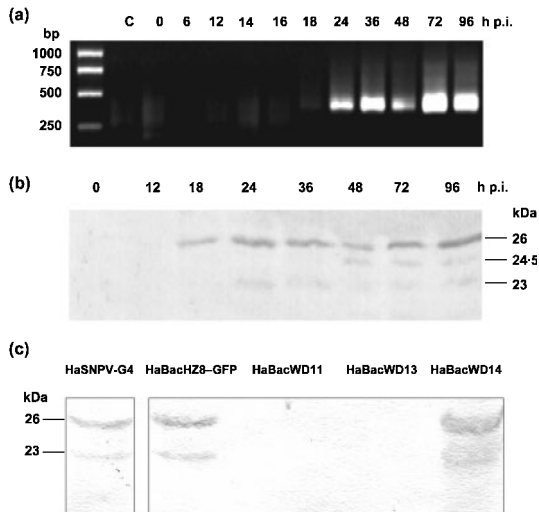


Fig. 2. (a) Analysis of transcription of the *fp25k* gene by 3'RACE. Total RNA samples were collected from HearNPV-G4-infected cells at different time points p.i. cDNA was synthesized and then used as template in PCR reactions. PCR products were analysed by agarose gel electrophoresis. Lane C: sample from non-infected cells. DNA ladder sizes are shown on the left side. (b) Expressional analysis of HearNPV *fp25k*. Cellular proteins were harvested from HearNPV-G4-infected cells at appropriated time points p.i., and separated with 10% SDS-PAGE. A polyclonal antibody against HearNPV FP25K was used for Western blot analysis. The different sizes of FP25K were marked on the right side. (c) Detection of *fp25k* expression of the recombinant viruses. Cells were infected at an m.o.i. of 5 with different viruses. Cellular proteins were harvested at 48 h p.i., and separated with 10% SDS-PAGE. The polyclonal antibody against HearNPV FP25K was used for Western blot analysis.

growth curves of BV are shown in Figure 3. Before 24 h p.i., the replication kinetics of the four viruses were very similar ($F_{3,11}=0.96$; $P=0.48$). After 24 h p.i., the *fp25k*-deleted

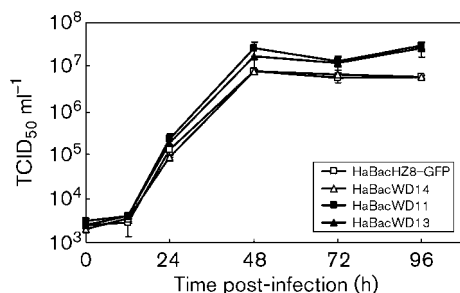


Fig. 3. BV growth curves of recombinant viruses. HzAM1 cells were infected with HaBacHZ8-GFP, HaBacWD11, HaBacWD13 or HaBacWD14, respectively. The BV titres were determined at appropriate time p.i. by the end-point dilution method. The growth curves were generated by arithmetic mean data of three infections.

viruses (HaBacWD11 and HaBacWD13) always had a higher BV titre than those of non-deleted virus (HaBacHZ8-GFP) and rescued virus (HaBacWD14) (Fig. 3), although the differences were not significant. At 96 h p.i., the production of BVs was more significant from *fp25k*-deleted viruses than that of the *fp25k*-containing viruses ($F_{3,11}=7.416$; $P=0.011$).

Electron microscopy of cells infected with the recombinant viruses

HaBacHZ8-GFP produced a large number of enveloped nucleocapsids that accumulated at the ring zone of the inner side of the nuclear membrane (Fig. 4a). Only a few

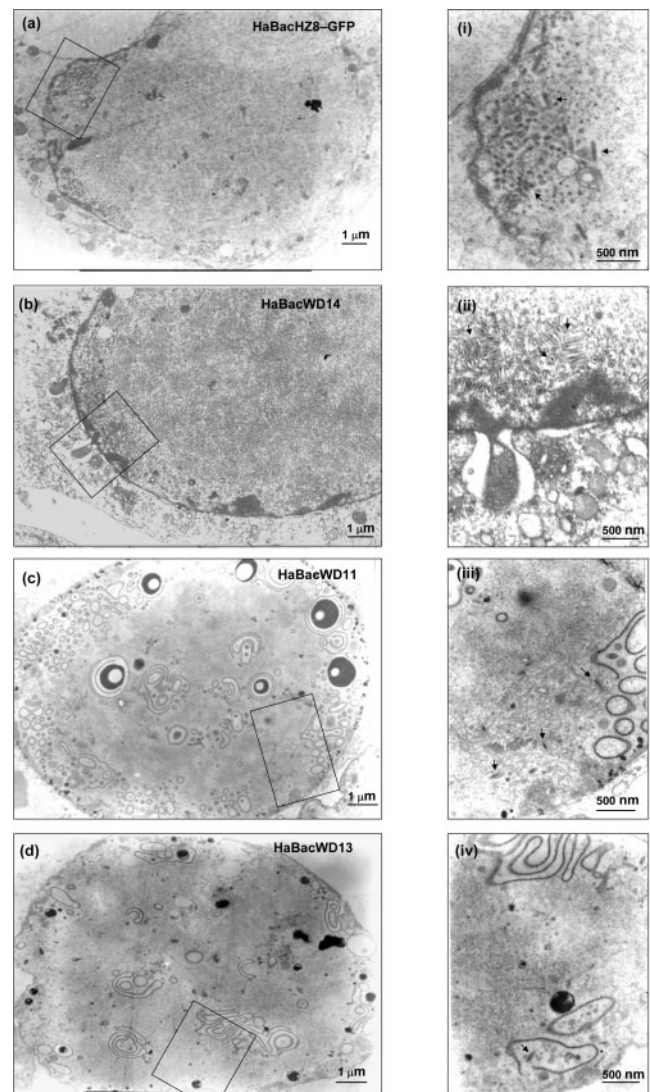


Fig. 4. Electron microscopy of cells infected with the recombinant viruses. HzAM1 cells were infected with HaBacHZ8-GFP (a, i), HaBacWD14 (b, ii), HaBacWD11 (c, iii) or HaBacWD13 (d, iv) at an m.o.i. of 5, respectively. Cells were fixed at 96 h p.i. (i-iv) Enlargements of the blocked area in (a-d), respectively. The arrows indicate virions.

nucleocapsids could be found in the nucleus of cells infected with HaBacWD11 and HaBacWD13, where FP25K was inactivated (Fig. 4c and d). The number of ODVs in cells infected with the rescued virus (HaBacWD14) (Fig. 4b) was similar to that generated by HaBacHZ8-GFP. In the infected cells, the mean number of ODV particles per cell section at 96 h p.i. was 170, 186, 29 and 31 for HaBacHZ8-GFP, HaBacWD14, HaBacWD11 and HaBacWD13, respectively.

Expression of Ha133 and ODV-E66 by different recombinants

H2AM1 cells were infected with HaBacHZ8-GFP and HaBacWD11. The infected cells were harvested at 0, 12, 18, 24, 48, 72 h p.i. Anti-HaF1 and anti-HaE66 antisera were used to detect the expression of Ha133 and ODV-E66, respectively (Fig. 5). In cells infected with each virus, Ha133 was detected from 12 to 72 h p.i. and ODV-E66 protein was detected from 48 to 72 h p.i. There was no observable difference in the expression between the two viruses.

DISCUSSION

In this manuscript, we reported on the transcription and expression of the *fp25k* in HearNPV, and have also reported on the properties of *fp25k*-null viruses. Transcription of HearNPV *fp25k* was initiated at 18 h p.i. The protein was detected at the same time with anti-FP25K antiserum. This is similar to that observed in AcMNPV-infected cells (Harrison & Summers, 1995a).

In cells infected with HearNPV, FP25K was expressed in two major molecular forms, 26 and 23 kDa (Fig. 2b and c), which is similar to the two forms in AcMNPV-infected Sf21 cells (Braunagel *et al.*, 1999). The smaller molecule may be the second product translated from the internal AUG codon present at 81 nt downstream of the first AUG codon in HearNPV. In AcMNPV, there is also an internal Met at aa 32 of FP25K. As not all sequenced baculovirus

fp25k genes contain the second internal ATG codon, the functional significance of the small protein has not yet been determined. A third molecular form of FP25K, with the size of 24.5 kDa, was detected in HearNPV-infected cells after 48 h p.i. (Fig. 2b), and it was not reported in AcMNPV-infected cells. The function of the 24.5 kDa protein is not known. We cannot rule out at present that the 24.5 kDa protein is a degradation product, even though protease inhibitors were used in the preparation of the infected-cell lysates.

Previously, most investigations on FP mutants were carried out on viruses with active polyhedrin. Since one of the effects of the FP25K mutation was decreased levels of polyhedrin mRNA together with an altered transport of polyhedrin into the nucleus, it raised the question if other effects of the FP25K mutation were the consequences of polyhedrin alteration. In this study, we used a polyhedrin-null bacmid system to study the function of FP25K. Our results indicate that FP25K mutants could cause increased production of BV and decreased amount of ODV production in the absence of a polyhedrin gene. It has been reported that AcFP β gal-CAT, a recombinant AcMNPV without polyhedrin and FP25K-negative, could release more infectious particles than Ac360CAT, which was occlusion-negative and FP25K-positive (Harrison & Summers, 1995b). Our data and those of Harrison & Summers (1995b) support each other with respect to the fact that the effects of FP25K mutants may not be related to polyhedrin.

HearNPV belongs to group II NPV (Chen *et al.*, 1999), which use a homologue of Ld130 as a membrane fusion protein instead of GP64 present in group I nucleopolyhedroviruses (Pearson *et al.*, 2000). The homologue of Ld130 in HearNPV is Ha133 (Chen *et al.*, 2001). It was reported that AcMNPV *fp25k* mutants had enhanced accumulation of GP64 and decreased accumulation of ODV-E66 (Braunagel *et al.*, 1999; Rosas-Acosta *et al.*, 2001). The decrease of AcMNPV ODV-E66 was regulated either directly or indirectly at the translation level by FP25K (Rosas-Acosta *et al.*, 2001). The deletion of FP25K also altered the transportation and localization of ODV-E66 during infection (Rosas-Acosta *et al.*, 2001). However, we did not observe significant changes in the level or temporal expression of Ha133 or ODV-E66 in HaBacWD11 (FP25K mutant)-infected cells in comparison to the wild-type parental virus HaBacHZ8-GFP. We suggest that the transportation and localization of ODV-E66 in FP25K-deleted mutants need to be investigated.

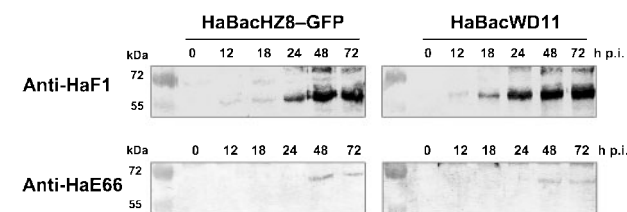


Fig. 5. Expression of F protein and ODV-E66 by different recombinants. Cells were infected at an m.o.i. of 5 with HaBacHZ8-GFP or HaBacWD11. Cellular proteins were harvested at appropriate time points p.i., and separated with 10% SDS-PAGE. About 2×10^3 cells were loaded in each lane. The polyclonal antibody against HearNPV Ha133 (anti-HaF1) and ODV-E66 (anti-HaE66) was used for Western blot analysis.

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