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# Assessment of foreign protein production by recombinant *Heliothis* (*Helicoverpa*) *armigera* entomopoxviruses in *Spodoptera frugiperda* cells

Julie A. Olszewski and David J. Dall

CSIRO Entomology, GPO Box 1700, Canberra ACT 2601, Australia

**This report describes the first production of recombinant forms of *Heliothis* (*Helicoverpa*) *armigera* entomopoxvirus (HaEPV). These HaEPVs are engineered at either the spheroidin or fusolin locus, to produce the green fluorescent marker protein (GFP). The growth properties of these recombinant HaEPVs, in comparison to the parental HaEPV, were assessed in cultured *Spodoptera frugiperda* Sf9 cells. Additionally, GFP production by these recombinant HaEPVs was compared to that of a GFP-expressing recombinant of the baculovirus *Autographa californica* nucleopolyhedrovirus (AcNPV) in the same *in vitro* system, at various multiplicities of infection. Expression of GFP from the HaEPV spheroidin locus produced up to 60% of that generated from the AcNPV polyhedrin locus, albeit over a longer period of infection. A considerably lower yield was recorded from the HaEPV fusolin locus, a result that contrasted markedly with the apparent activity of this promoter in caterpillar infections *in vivo*. The potential applications for further development of HaEPV expression systems are discussed.**

## Introduction

Members of the poxvirus family (*Poxviridae*) collectively infect a wide range of vertebrates and insects, and this disjunct host distribution provides the basis for division of the family into the subfamilies *Chordopoxvirinae* and *Entomopoxvirinae* (Murphy *et al.*, 1995). Entomopoxviruses (EPVs) have been described from a spectrum of hosts that includes grasshoppers, beetles, midges, cockroaches (Radek & Fabel, 2000) and caterpillars. *Heliothis* (*Helicoverpa*) *armigera* entomopoxvirus (HaEPV) is a lepidopteran-infecting EPV (Goodwin *et al.*, 1991).

While all members of the *Poxviridae* possess a series of conserved fundamental characteristics such as genome structure and virion morphology, EPVs distinctively and characteristically incorporate their mature intracellular virions into large proteinaceous occlusion bodies known as spheroids (Arif, 1995). These structures are believed to reduce the impact of environmental stresses on the occluded virus particles following their release after death of the host, and are thus assumed to function in a manner analogous to counterparts termed polyhedra, produced by insect-infecting members of the baculovirus and reovirus families (Miller, 1996).

**Author for correspondence:** Julie Olszewski. Present address: Department of Biological Sciences, Imperial College of Science, Technology, and Medicine, Imperial College Road, London SW7 2AZ, UK. Fax +44 207 584 2056. e-mail j.olszewski@ic.ac.uk

The most abundant component of the EPV spheroid is a high molecular mass (100–120 kDa) matrix protein known as spheroidin (SPH), which is apparently uniquely associated with this group of viruses (King *et al.*, 1998). SPH from HaEPV has a deduced relative molecular mass of 115 438, and is relatively closely related to homologues from other lepidopteran-associated EPVs, showing amino acid identities of 82 and 79% to SPH proteins from *Amsacta moorei* EPV (AmEPV) and *Choristoneura fumiferana* EPV (Sriskantha *et al.*, 1997). SPH is abundantly expressed by HaEPV in both *in vivo* and *in vitro* infections (Dall *et al.*, 1993; J. A. Olszewski & D. J. Dall, unpublished). Similarly, studies of AmEPV *in vitro* have shown that SPH is the most abundantly expressed viral protein in infected cells (Winter *et al.*, 1995) and, in addition, that its synthesis is not essential to virus replication (Palmer *et al.*, 1995).

Recognition of these characteristics led to suggestions that the AmEPV spheroidin locus (*sph*) might present a valuable site to develop a system for expression of heterologous proteins (Palmer *et al.*, 1995; Li *et al.*, 1998), in a manner similar to that previously developed for the prototypic poxvirus and baculovirus, vaccinia virus and *Autographa californica* nucleopoly-

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**Table 1.** Oligonucleotides used for experimental procedures described in text

| Oligonucleotide | Sequence |
|---|---|
| TV497A | 5′ CCGAATTCGGTTTATTAGAATCGGTC 3′ |
| TV497B | 5′ TTAGATCTCTCGAGCGTCTCTTTATTATTATTAATATATAAAAA 3′ |
| TV497C | 5′ AATCTAGAAACTTTCATTCATTAATTTTG 3′ |
| TV497D | 5′ ATAAGCTTATAAAACCGAATTAGGAGG 3′ |
| Oligo-RS | 5′ CTACTTTAACTTATGGTGTTC 3′ |
| GFXB4 | 5′ AAACTCGAGCGTCTCAATGAGTAAAGGAGAAGAAC 3′ |
| GFX2 | 5′ AATTCTAGAATGCTATTTGTATAG 3′ |
| TV2962A | 5′ GAACAAAATACCTTGATGG 3′ |
| TV697.1 | 5′ TACTCGAGCGTCTCTTATTAATCTTTGG 3′ |
| TV2962C | 5′-TCGATCTAGATTTTTTTCGGGAGTATGGATTATAATACG 3′ |
| TV2962D | 5′ GATTGAAAGCTTATATCATCTGCAC 3′ |
| TV497E | 5′ ATCCTTGTACTATACCGTC 3′ |
| A21R1A | 5′ GGTTGATCTATATGTTTGGG 3′ |
| EPSP13 | 5′ ATTACTATTCTGTGCTGAAGG 3′ |
| EPSP16 | 5′ CAAATTGAGCAGGTCTTACG 3′ |

hedrovirus (AcNPV) (Mackett *et al.*, 1982; O'Reilly *et al.*, 1992). In the case of HaEPV, this possibility was further enhanced by observations that the virus could replicate in a non-lytic manner in serum-free cultures of lepidopteran cells (D. J. Dall, unpublished), potentially simplifying the purification of foreign proteins secreted into the culture medium. Additionally, HaEPV replicates in cells which have been widely used for baculovirus expression systems, such as *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* 'Hi-5' cells (Invitrogen).

Initial experiments on the AmEPV *sph* locus suggested that it may be less productive than originally envisaged. Palmer *et al.* (1995) reported that expression of chloramphenicol acetyltransferase from the AmEPV *sph* locus in *Lymantria dispar* cells occurred at levels of only 5–10% of those observed when the same protein was expressed in a baculovirus system under the polyhedrin (*polh*) promoter. Subsequently, however, it was reported that the activity of the promoter could be substantially improved by strict conservation of the native TAAATG consensus nucleotide sequence at the translation initiation codon (King *et al.*, 1998). Further to this observation, Li *et al.* (1998) demonstrated that high-level expression of β-galactosidase from the AmEPV *sph* promoter was dependent on retention of native *sph* coding sequence from the 5′ region of the gene, although the fact that expression was from a heterologous genomic locus makes the general validity of this observation more difficult to assess. Taken together, these findings provide only limited support for further development of AmEPV as a protein expression system; nevertheless, the possibility that *sph* loci of other EPV isolates might possess more favourable capabilities remains to be examined.

Furthermore, AmEPV is unusual among lepidopteran EPVs in lacking the locus that encodes the viral fusolin (FUS) protein. This polypeptide is the primary constituent of EPV-associated 'spindle bodies' (Dall *et al.*, 1993; Lai-Fook & Dall, 2000), and is the most abundant protein in preparations of HaEPV originating from *in vivo* infections. Homologues of this protein are also found in many baculoviruses, and are generally referred to as gp37 (Phanis *et al.*, 1999). Gp37 synthesis has recently been inactivated in AcNPV and thus been shown to be non-essential for baculovirus replication (Cheng *et al.*, 2001). The observation that neither of two fully sequenced EPVs possess copies of the *fusolin* (*fus*) gene (Afonso *et al.*, 1999; Bawden *et al.*, 2000) suggested that the protein is also unlikely to be essential for replication of HaEPV. On this basis we considered that the HaEPV *fus* locus might provide another strong viral promoter from which heterologous gene products could be expressed.

In this paper we report the engineering of HaEPV, using a 'seamless' cloning strategy, to exactly replace either the *sph* or *fus* gene with nucleotide sequence encoding the green fluorescent protein (GFP; Chalfie *et al.*, 1994). We examine the growth kinetics of the recombinant HaEPVs in Sf9 insect cells in culture, report kinetics of GFP production by the recombinants, and compare these with those of a recombinant AcNPV baculovirus, in which production is controlled by the *polh* promoter.

## Methods

■ **Virus and cells.** Clonal wild-type (wt) isolate HaEPV wt#2/011293 (WT2; Osborne *et al.*, 1996), and its derivatives, HaEPV(sph−) and HaEPV(fus−), were propagated in Sf9 (ATCC CRL1711) or *Helicoverpa zea* (BCIRL-Hz-AM1; McIntosh & Ignoffo, 1981) cells as previously described (Fernon *et al.*, 1995). Viral stocks were maintained at fewer than 10 passages; infections were propagated by incubation of plated cells with infectious media for 2 h before removal of inoculum and replacement with fresh medium containing 10% heat-inactivated bovine calf serum. Baculovirus BacPAK 6 (Clontech) and its derivative BacPAK-GFP(RS) were similarly propagated in Sf9 cells; stocks were maintained at passage

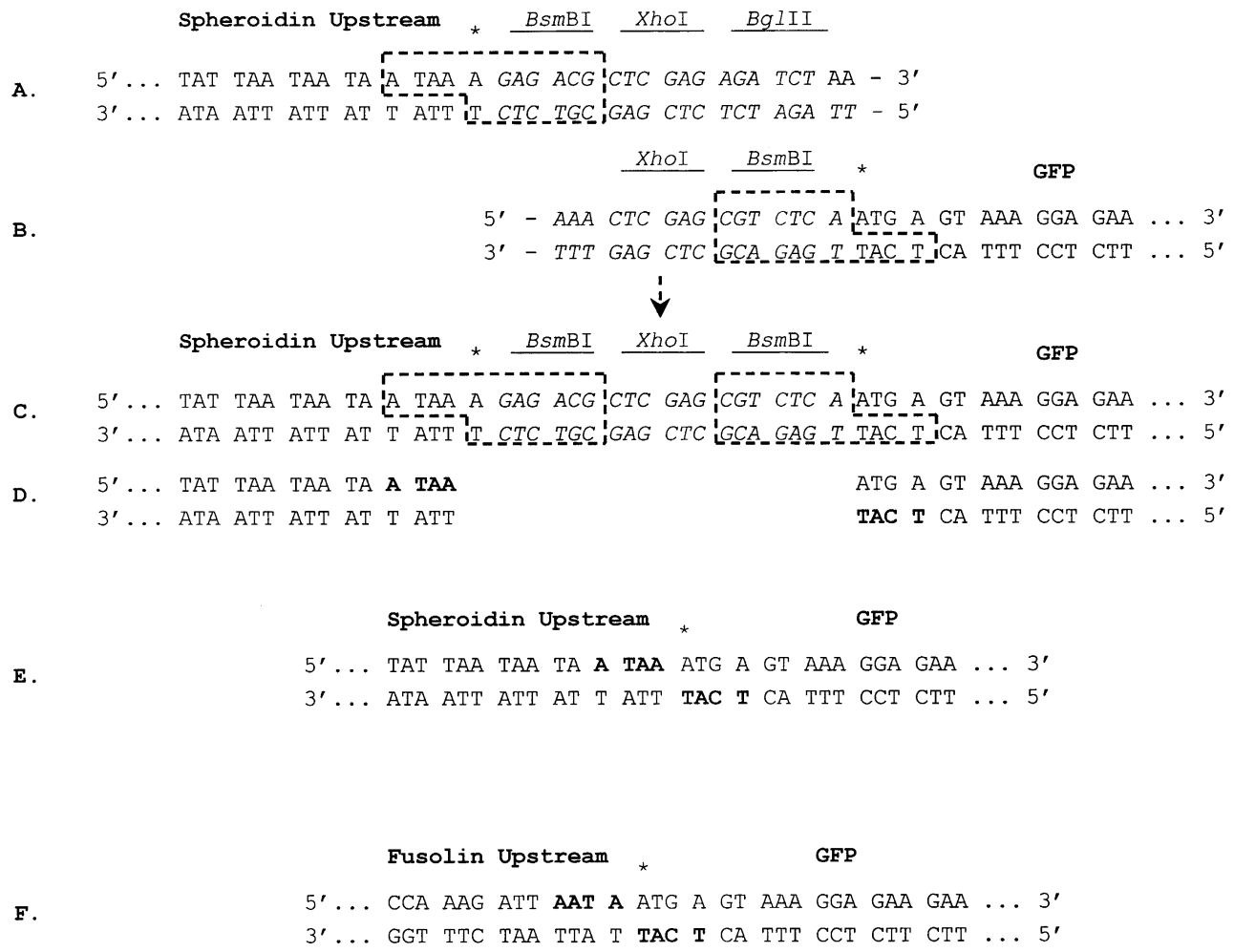


Fig. 1. 'Seamless' cloning of *gfp*(RS) with HaEPV promoters. (A) Schematic representation of nucleotide sequence directly 'upstream' of the ATG of the *spheroidin* gene, to which additional sequence (italics), including *Bsm*BI, *Xho*I and *Bgl*II sites, was added by PCR (see Methods) to facilitate cloning. The non-palindromic degenerate *Bsm*BI recognition sequence is indicated (box) and an asterisk denotes the first nucleotide of the *spheroidin* coding sequence. (B) Representation of the 5′ end of the *gfp*(RS) gene to which additional sequence (italics) was added; this included an *Xho*I site, and a *Bsm*BI site in an orientation opposite to that inserted in the construct shown in (A). Cloned sequences depicted in (A) and (B) were subsequently joined (broken arrow) at the *Xho*I sites (C), digested with *Bsm*BI and filled in [shown in bold; (D)] and then re-ligated (E). The same methodological approach was used to join the HaEPV *fusolin* promoter to the *gfp*(RS) gene (see Methods), producing the promoter/coding junction shown in (F).

3. Viral infectious titres were quantified by the $TCID_{50}$ method (Reed & Muench, 1938).

■ **Plasmid constructs.** About 0·5 μg or 10 ng of viral genomic or plasmid DNA, respectively, was used as template for PCR amplifications using *Taq* DNA polymerase (GibcoBRL). Reaction cycles used a template denaturation step of 5 min at 94 °C, followed by 36 cycles of 94 °C for 1 min, 45 to 55 °C for 1 min, and 72 °C for 1 min. PCR products were cloned and their sequence verified using automated DNA sequencing.

A 'seamless' cloning strategy was employed to make the transfer vector pTV497, subsequently used for replacement of the HaEPV *sph* gene (GenBank accession no. AF019224) with a modified version (see below) of the *Aequorea victoria gfp* gene. An HaEPV region 'upstream' of the *sph* gene (−1 to −797) was PCR amplified from wt HaEPV genomic DNA with primers TV497A and TV497B (Table 1). These primers added an *Eco*RI restriction site at the 5′ end of the amplified fragment, and *Bsm*BI, *Xho*I and *Bgl*II restriction sites at the 3′ end (Fig. 1A). After co-digestion with *Eco*RI and *Bgl*II this fragment was cloned into *Eco*RI/*Bam*HI-linearized pTZ19R vector plasmid (AmershamPharmacia) to create pTV497-UI2. A 1400 bp region 'downstream' of the spheroidin translation termination codon was amplified from wt HaEPV genomic DNA with primers TV497C and TV497D (Table 1), adding *Xba*I and *Hin*dIII sites at the 5′ and 3′ ends, respectively, of the product. After digestion this product was cloned into *Xba*I/*Hin*dIII-digested pTV497-UI2 to create pTV497-I.

A brighter red-shifted (RS) variant of the GFP from *A. victoria* was produced by mutagenizing the codons for F64 and S65 to encode leucine and threonine residues, respectively (Cormack *et al.*, 1996). The variant was constructed by oligonucleotide-directed mutagenesis of the wild-type nucleotide sequence (Chalfie *et al.*, 1994), using an oligonucleotide designated Oligo-RS (Table 1) and the modified Kunkel method (Sambrook *et al.*, 1989). Successful incorporation of the mutations was confirmed by DNA sequencing of the product. PCR amplification of this

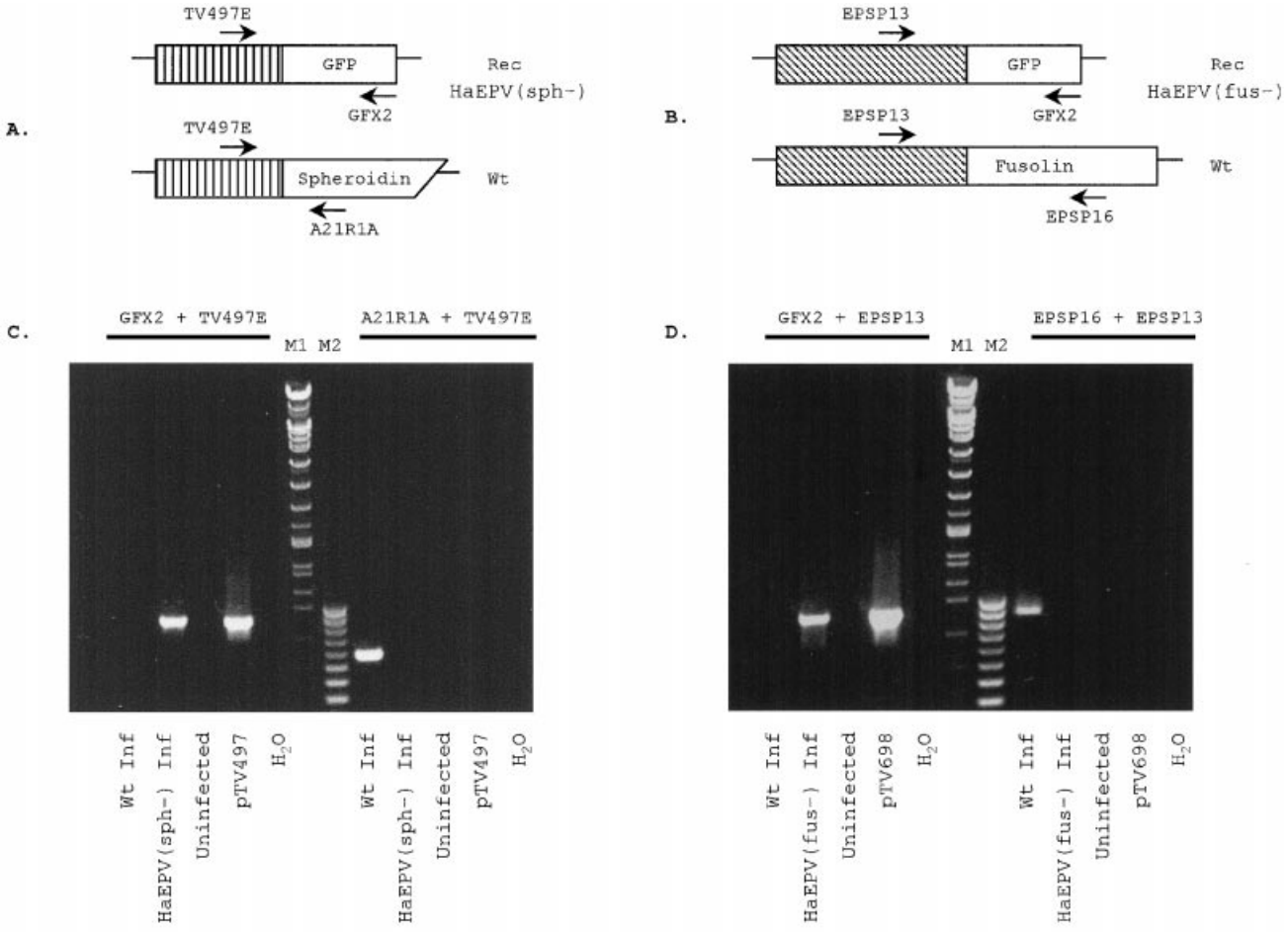


Fig. 2. Analysis of recombinant virus isolates. (A) Schematic representation of the *spheroidin* locus with oligonucleotide primer binding sites indicated (arrows) for HaEPV(sph−) (top), and wt HaEPV (bottom). Protein coding sequence is represented by open boxes and sequence upstream of *spheroidin* is shown with hatched boxes. (B) Representation of the *fusolin* locus with primer binding sites for HaEPV(fus−) (top), and wt HaEPV (bottom); cross-hatched boxes represent sequence upstream of *fusolin*. (C) Electrophoretic analysis of products of PCR reactions from recombinant virus isolates. DNA markers (M1 and M2) are 'Hi-Lo'(Bresatec) and 100 bp ladder (Life Technologies), respectively; only 300–1000 bp range (fragments are in 100 bp increments) of M2 is shown. The left half of the panel shows PCR products from primers (indicated above panel) specific for the recombinant *spheroidin* locus, used with (from left to centre) wt HaEPV-infected medium, HaEPV(fus−)-infected medium, uninfected medium, pTV497 transfer vector plasmid or water control. The right panel shows PCR products from the same templates, using primers specific to the wt HaEPV *spheroidin* locus. (D) PCR screening of templates as in (C), except that the recombinant-infected medium contained isolate HaEPV(fus−), and the transfer vector plasmid was pTV698. The left half of the panel shows PCR products from primers (indicated above) specific to the recombinant *fusolin* locus, and the right half products for those primers specific to the wt HaEPV *fusolin* locus.

mutated sequence with primers GFXB4 and GFX2 added *Xho*I and *Bsm*BI sites at the 5′ end of the fragment (Fig. 1B), and an *Xba*I site at the 3′ end. These restriction enzyme sites facilitated cloning of the 750 bp product into the *Xho*I and *Xba*I sites of pTV497-I (Fig. 1C). Finally, joining of the *sph* upstream sequence with the ATG of the *gfp*(RS) sequence was accomplished by digestion of this plasmid with *Bsm*BI, fill-in of 5′ overhanging nucleotides with T4 DNA polymerase (NEB) and dNTPs (Fig. 1D), and re-ligation (Fig. 1E). All cloning junctions in the resulting transfer vector pTV497 were confirmed by DNA sequencing.

A corresponding strategy was employed to insert the same *gfp*(RS) gene under control of the HaEPV *fus* promoter. Construction of the transfer vector pTV698, used to replace the HaEPV *fus* gene (GenBank accession no. L08077) with the *gfp*(RS) gene, entailed PCR amplification of the region upstream of the *fus* gene, using wt HaEPV genomic DNA and oligos TV2962A and TV697.1 (Table 1). This procedure introduced *Bsm*BI and *Xho*I sites at the 3′ end of the resulting 1·08 kb product. The product was purified (GeneClean; Bio101), end-filled and kinased using DNA Pol I and T4 polynucleotide kinase (GibcoBRL), following manufacturers' protocols. The resultant fragment was cloned into *Sma*I-digested pTZ19R, and inserted products were screened for correct orientation. A clone with the 3′ end of the inserted sequence proximal to the *Xba*I site of the parental vector was selected, digested with *Xho*I and *Xba*I, and a *Xho*I–*Xba*I-derived *gfp*(RS) fragment, as described above, was inserted, creating plasmid pFusPR-GFP. The region downstream of the HaEPV *fus* locus was amplified from wt HaEPV genomic DNA using oligos TV2962C and TV2962D (Table 1), introducing *Xba*I and *Hin*dIII sites at the 5′ and 3′ ends, respectively, of the 1·25 kb fragment. This *Xba*I–*Hin*dIII product was cloned into similarly digested pFusPR-GFP,

and joining of the *fus* promoter and the GFP(RS)-encoding sequence was achieved as described above, producing the vector pTV698 (Fig. 1F).

The transfer vector BacPAK8/GFP(RS), expressing *gfp*(RS) under control of the AcNPV *polh* promoter was constructed by inserting the *Xho*I–*Xba*I *gfp*(RS) fragment into the BacPAK8 vector (Clontech).

■ **Isolation and purification of recombinant viruses.** Recombinant HaEPVs were generated by transfecting transfer vector plasmid DNAs into Sf9 cells using DOTAP reagent (Roche) and standard protocols (O'Reilly *et al*., 1992) and then, after 18 h, infecting the same cells with wt HaEPV. Media was collected from the transfection/infections 5 days later and used to infect other Sf9 cells. Cells infected with recombinant HaEPVs were selected using fluorescence activated cell sorting (FACS) and introduced into six-well plates that had been lightly seeded with uninfected Sf9 cells. Several rounds of alternating FACS and plaque purification or end-point dilution (O'Reilly *et al*., 1992) were required before a stock free of contaminating parental wt HaEPV, as judged by PCR analysis (see below), was obtained for each recombinant virus.

The BacPAK-GFP(RS) virus was produced by co-transfecting the BacPAK8/GFP(RS) transfer vector with BacPAK6 viral DNA, previously digested with *Bsu*36I, according to the manufacturer's protocols (Clontech). Plaques which contained fluorescent cells were picked, amplified, and subjected to a second round of plaque purification. Verification of the recombinant junction at the *polh* locus was carried out by PCR analysis using primers Bac1 and Bac2 (Clontech).

■ **PCR screening of virus isolates.** PCR was used to screen for the presence of parental and recombinant HaEPV genomes following amplification in cell culture. Culture medium (25 µl) of was digested with 25 µl Proteinase K (400 µg/ml in 20 mM Tris, pH 8·0, 10 mM EDTA and 0·5% SDS) at 37 °C. Samples were boiled for 10 min, and then diluted with 200 µl $dH_2O$. PCR amplifications used 5 µl of this material as template. Stocks of recombinant viruses were analysed with two primer pairs in order to confirm the presence of the expected recombinant, and absence of parental virus (Fig. 2A, B).

Stocks of recombinant HaEPV(sph−) were analysed with primers GFX-2 and TV497E (Table 1), which anneal at the 3′ end of the GFP coding sequence, and to HaEPV genomic sequence upstream of the *sph* locus, respectively (Fig. 2A), to confirm the presence and context of the marker gene. The presence of contaminating parental HaEPV was assessed with primers TV497E and A21R1A (Table 1); the latter anneals within the HaEPV *sph* coding sequence. Stocks of HaEPV(fus−) were screened with primers GFX-2 and EPSP13 (Table 1), the latter of which anneals to genomic sequence upstream of the *fus* locus, and EPSP13 and EPSP16, the latter of which anneals to sequence within the *fus* locus. Positive controls were used in parallel with all reactions; templates were plasmids pTV497 and pTV698 for each engineered locus sequence, and wt HaEPV-infected medium for parental sequences. Mock-infected cell culture medium and $dH_2O$ served as templates in various negative control reactions. PCR entailed 35 cycles of: 1 min, 94 °C, 2·5 min, 45 °C, and 1·5 min, 72 °C. PCR products were assessed by agarose gel electrophoresis.

■ **Time-course of extracellular virus production.** Sf9 cells (7500 per well) were seeded in 96-well tissue culture-treated microtitre plates (Falcon) and allowed to attach. Infections were established, as described above, for wt HaEPV, HaEPV(fus−) and HaEPV(sph−), at m.o.i. values of 0·1, 0·5 and 2·0 infectious particles per cell. Inocula were carefully removed after 2 h and replaced with fresh medium. At various time-points (time zero being the time at which the infectious inoculum was added) cells and media were collected from duplicate wells for each viral inoculum per m.o.i. Cells were pelleted by microcentrifugation (2000 r.p.m., 5 min) and supernatants were transferred to clean tubes and stored at −80 °C until all samples had been collected. The amount of infectious virus present in each sample was quantified by $TCID_{50}$ analysis.

■ **Analysis of GFP production by fluorimetry.** Sf9 cells (5000 per well) were seeded in alternating wells of 96-well plates and allowed to attach. Cells were mock-infected or infected with HaEPV(sph−), HaEPV(fus−) or BacPAK-GFP(RS) at m.o.i. values of 0·1, 0·5 or 2·0, in quadruplicate, as described above. GFP fluorescence was either measured directly from these infected plates, or media and cells were collected, then diluted and measured in parallel in microtitre plates. To estimate the quantity of GFP produced by each infection on the basis of its measured fluorescence intensity, parallel measurements were made on a dilution series (50 to 1500 ng per well) of purified recombinant red-shifted EGFP (Clontech) prepared in medium containing uninfected cells. Fluorescence was measured with a FLUOstar fluorimeter (BMG LabTechnologies) using a standard fluorescence reading head, an excitation filter of 485 nm and an emission filter of 520–535 nm, 10 flashes per well with the gain set against the greatest quantity EGFP standard.

## Results

### Validation of recombinant virus stocks

A PCR screening strategy was employed to confirm that the *gfp*(RS) gene had been introduced at the correct locus in the putative *sph* replacement virus HaEPV(sph−), and that the cloned recombinant isolate was free of contaminating parental virus. Primers TV497E and GFX2 (Table 1 and Fig. 2A, upper), which anneal upstream of *sph* coding sequence and at the 3′ end of *gfp*(RS) coding sequence, respectively, were used to detect the presence of an appropriate recombinant sequence at the *sph* locus in the virus stock. Primers TV497E and A21R1A, the latter of which anneals to the *sph* coding sequence, were used to check for the presence of virus carrying the parental sequence at the *sph* locus (Table 1 and Fig. 2A, lower). As expected, primer pair TV497E/GFX2 amplified a product of appropriate size (920 bp) from the transfer vector pTV497 (Fig. 2C, left panel), and also from medium from cultures of cells infected with the HaEPV(sph−) recombinant isolate; it did not amplify any product from media harvested from wt virus-infected cells or uninfected cells, or from a water control. In contrast, primer pair TV497E/A21R1A amplified a product of the expected size (580 bp) from medium from wt HaEPV-infected cells, but not from medium from cells infected with the HaEPV(sph−) isolate (Fig. 2C, right panel). Additional characterization studies (data not shown) used Southern blot analysis to compare restriction profiles of genomic DNA from wt virus and HaEPV(sph−) virus stocks; the only observed differences were in fragments containing the *sph* locus. These data indicate that a recombinant form of HaEPV, in which *sph* coding sequence was replaced by that of *gfp*(RS), had been produced and isolated. This result shows that expression of the HaEPV *sph* gene is not essential for virus replication in cells in culture, in agreement with observations made with AmEPV (King *et al*., 1998).

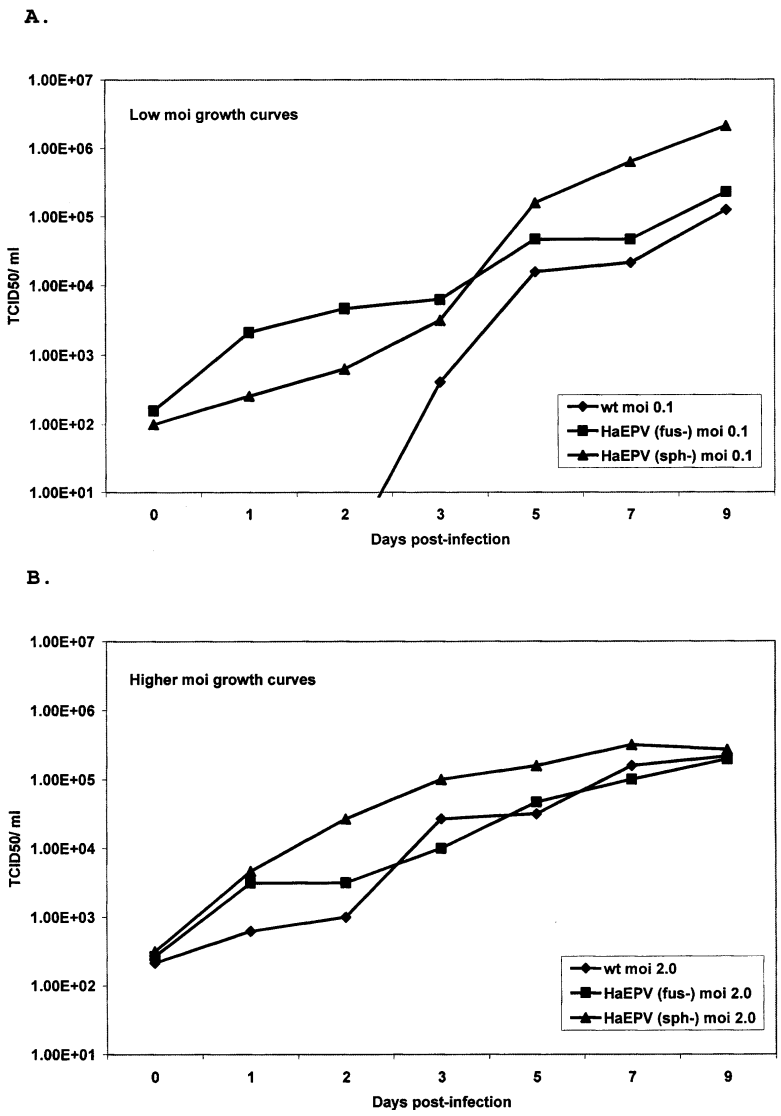


Fig. 3. Production of ECV by recombinant HaEPV isolates. Sf9 cells were infected at a multiplicity of 0·1 (A) or 2·0 (B) with wt HaEPV (wt; ◆), HaEPV(fus−) (■) or HaEPV(sph−) (▲). At the indicated times post-infection, media were collected and titres of infectious extracellular virus were determined. These data are from one of two independent experiments with similar results.

A PCR strategy was also used to screen isolates of HaEPV putatively engineered at the *fus* locus. Thus, primer EPSP13 (Table 1), which anneals to sequence upstream of the *fus* locus, was used in conjunction with primer GFX2 to amplify a product of 850 bp from both the pTV698 transfer vector plasmid and the recombinant virus isolate HaEPV(fus−) (Fig. 2B, upper, and 2D, left panel). The corresponding DNA fragment was not amplified from media collected from wt HaEPV-infected cell cultures or from uninfected cells, or from water only negative control reactions (Fig. 2D, left panel). In contrast, primer pair EPSP13/EPSP16 (Table 1) identified the presence of virus carrying the parental *fus* locus in media from wt-infected cells (Fig. 2B, bottom, and 2D, right panel). Amplification of this 950 bp product was not observed in reactions where media from HaEPV(fus−)-infected cells were used as template, or in other control reactions (Fig. 2D). These data show that a recombinant form of HaEPV, in which the *fus* coding sequence was replaced by that of *gfp*(RS), had been successfully isolated. This is the first demonstration of deletion of an endogenous *fus* coding sequence from an EPV, and shows that this gene is non-essential for growth of HaEPV in cell culture.

## Kinetics of extracellular virus production by recombinant forms of HaEPV

The kinetics of extracellular virus (ECV) production of HaEPV in cell culture have not previously been described. In work reported here we assayed temporal and quantitative aspects of the process by determining the infectious titre of media harvested from cultures of Sf9 cells infected with either wt or recombinant forms of the virus. As shown in Fig. 3(A), ECV progeny of wt HaEPV from infections established at an m.o.i. of 0·1 were first detectable 3 days post-infection (p.i.),

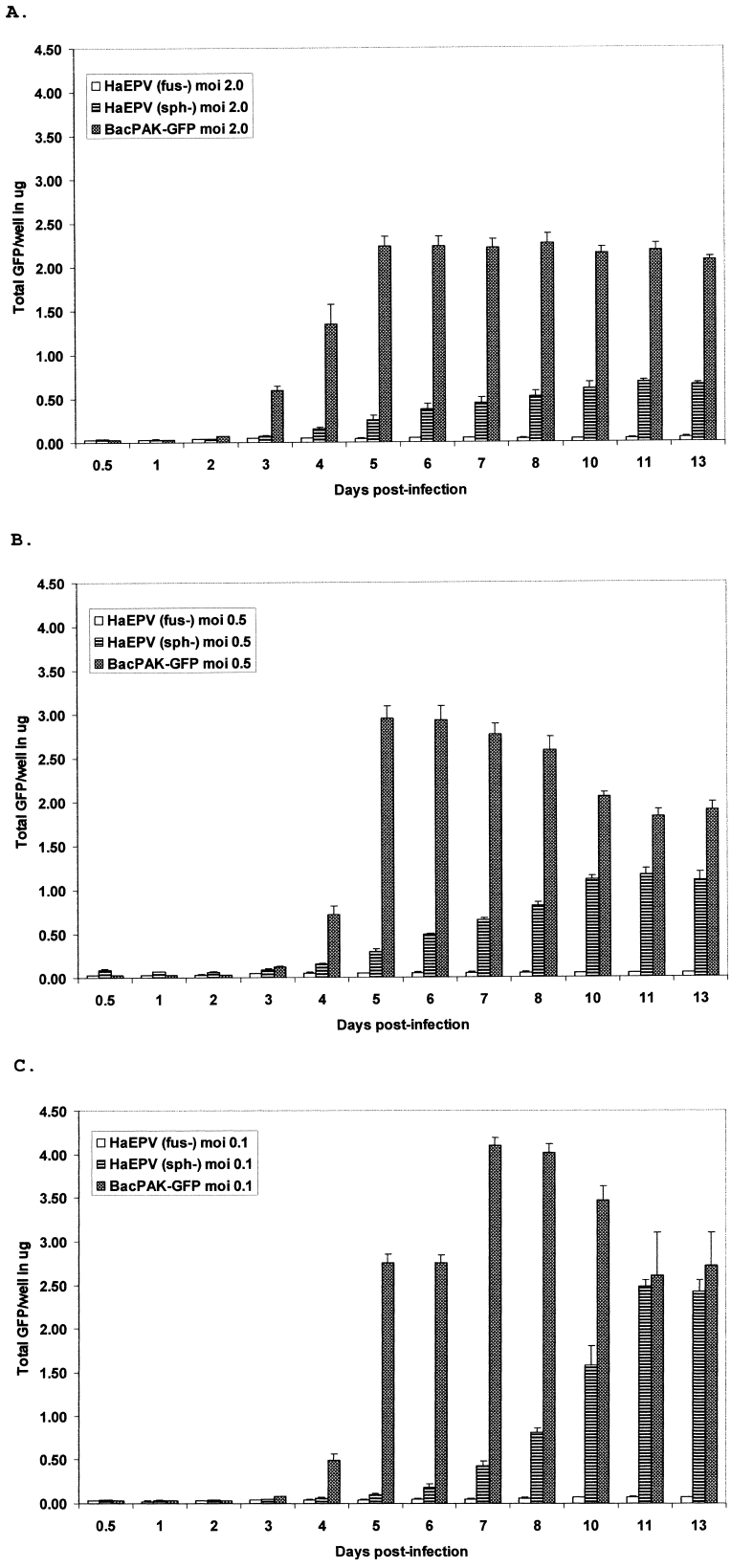


Fig. 4. GFP production by recombinant HaEPVs and a recombinant baculovirus. Sf9 cells were infected with HaEPV(fus−) (open bars), HaEPV(sph−) (striped bars) or BacPAK-GFP(RS) (BacPAK-GFP; solid bars) at an m.o.i. of 2·0 (A), 0·5 (B) or 0·1 (C). Quantities of GFP produced at the indicated times post-infection were determined by fluorimetry and conversion of fluorescence units to μg GFP by comparison to a purified protein curve.

and had increased substantially in concentration at 5 days p.i., when the recorded titre was $1{\cdot}58 \times 10^4$ $TCID_{50}$ units/ml. ECV continued to accumulate, albeit more slowly, between 5 and 9 days p.i., and ultimately achieved a titre of $1{\cdot}26 \times 10^5$ $TCID_{50}$ units/ml. The experiment was concluded at 9 days p.i., on the basis of data from pilot experiments (not shown) that suggested that significantly higher ECV titres are not achieved past this time.

ECV production by parallel infections of HaEPV(sph−) and HaEPV(fus−) was determined using the same assay (Fig. 3A). At time zero a titre of about 100 infectious units/ml was detected for each recombinant virus; this material is presumed to represent input virus not taken up during the initial infection period, but subsequently carried over into the replacement medium (see Methods). Other studies have shown that wt HaEPV DNA replication is not detectable until several hours after this time (D. Carpentier & J. A. Olszewski, unpublished data). ECV from recombinant-infected cells accumulated at a relatively uniform rate over the 9 days of the experiment (Fig. 3A), with neither recombinant showing the apparent rapid onset of productivity observed for the parental virus, as described above. We consider this apparent difference in kinetics to be artefactual, likely reflecting the relative ease of screening GFP-producing infections at very low levels as compared to wt HaEPV infections.

Kinetics of ECV production were also assessed for infections established at the higher m.o.i. of 2·0. The most apparent consequence of this 20-fold increase in inoculum concentration was the presence of higher titres of progeny ECV at early times of infection, an effect that was particularly evident for the wild-type parental form. Assays at later stages of infection (5–9 days p.i.) showed similar levels of progeny production for all three forms of the virus, with observed maxima between 1·95 and $2{\cdot}69 \times 10^5$ $TCID_{50}$ units/ml (Fig. 3B). These results indicate that replacement of either of the HaEPV *sph* or *fus* genes with one encoding GFP does not result in substantive alteration in the kinetics or amount of ECV production, and by extrapolation, that it is also unlikely to affect any fundamental parameters of virus replication in cell culture.

### Production of GFP by recombinant forms of HaEPV

In order to compare the *sph* and *fus* promoters with each other, and with the *polh* promoter driving expression of the same gene in the context of baculovirus infection, we established parallel infections of Sf9 cells with three recombinant insect viruses: HaEPV(fus−), HaEPV(sph−) and BacPAK-GFP(RS). Infections were established at three m.o.i. values – 2·0, 0·5 and 0·1 (Fig. 4A, B, C, respectively) and total GFP produced was measured from 0·5 to 13 days p.i., using a fluorescent plate reader. At an m.o.i. of 2·0, GFP expression from BacPAK-GFP(RS) could first be detected at 2 days p.i., and levels of GFP rose rapidly between 2 and 5 days p.i. to a maximum output of 2·27 μg GFP per well (Fig. 4A, solid bars). GFP amounts did not increase after 5 days p.i., and remained essentially constant until a slight decrease was noted at 13 days p.i. Therefore, from 5 to 13 days p.i. either little *de novo* synthesis of GFP occurred, or the rate of production closely matched the protein's turnover rate (Fig. 4A). In contrast, GFP produced by HaEPV(sph−) infections at an m.o.i. of 2·0 accumulated more gradually, with a maximum of 0·68 μg GFP per well being measured at 11 days p.i. (Fig. 4A, striped bars). Under these experimental conditions, the HaEPV *sph* promoter thus produced about 30% as much foreign protein as the *polh* promoter. Surprisingly, GFP expression from HaEPV(fus−)-infected cells was observed to be much lower than that from cells infected with either of the other two recombinants at the same m.o.i. Under these experimental conditions maximum GFP production was recorded at 0·05 μg GFP per well, and was attained at 11–13 days p.i. (Figs 4A and 5, open bars). The amount of GFP produced by activity of the *fus* promoter in this system was thus about 7% of that produced by the *sph* promoter.

In this same assay, lowering of the m.o.i. to 0·5 or 0·1 resulted in production of higher total amounts of GFP by all three recombinants (Figs 4B, C and 5). It is presumed that this reflects the fact that cells that initially escape infection continue to divide and therefore provide more target host cells for secondary infection. Thus, in cells infected with BacPAK-GFP(RS), maximum GFP production was recorded at 5 days p.i. (m.o.i. 0·5; Fig. 4B, solid bars) and 7 days p.i. (m.o.i. 0·1; Fig. 4C, solid bars), and corresponded to 2·95 μg and 4·10 μg GFP per well, respectively. In cells infected with HaEPV(sph−), maximum GFP production was recorded at 11 days p.i. for both m.o.i. values (m.o.i. 0·5; Fig. 4B, m.o.i. 0·1; Fig. 4C, striped bars), and corresponded to 1·16 μg and 2·49 μg GFP per well, respectively. Thus, for infections initiated at either 0·5 or 0·1, the HaEPV *sph* promoter directed synthesis of 40–60% of the amount of GFP generated by the AcNPV *polh* promoter.

Closer inspection of the kinetics of GFP production from HaEPV(fus−)-infected Sf9 cells (Fig. 5) showed that accumulation essentially plateaued after 4 days p.i. for infections initiated at an m.o.i. of 0·5 (Fig. 5, grey bars), but continued to increase until about 11 days p.i. for infections initiated at an m.o.i. of 0·1 (Fig. 5, black bars). In these assays maximum accumulation was measured as being 0·06 and 0·08 μg GFP per well, respectively. Once again, these levels of production were substantially below those of either of the other two recombinants assayed under the same conditions, representing about 2% and 3–5% of the corresponding amounts of GFP produced by BacPAK6-GFP(RS) and HaEPV(sph−), respectively. Nevertheless, like both other recombinants, HaEPV(fus−) produced the most GFP from infections initiated at an m.o.i. of 0·1, and took correspondingly longer to reach this maximum. On the basis of these observations, and the ECV studies reported above, we consider that the observed low level of accumulation of GFP reflects HaEPV *fus* promoter

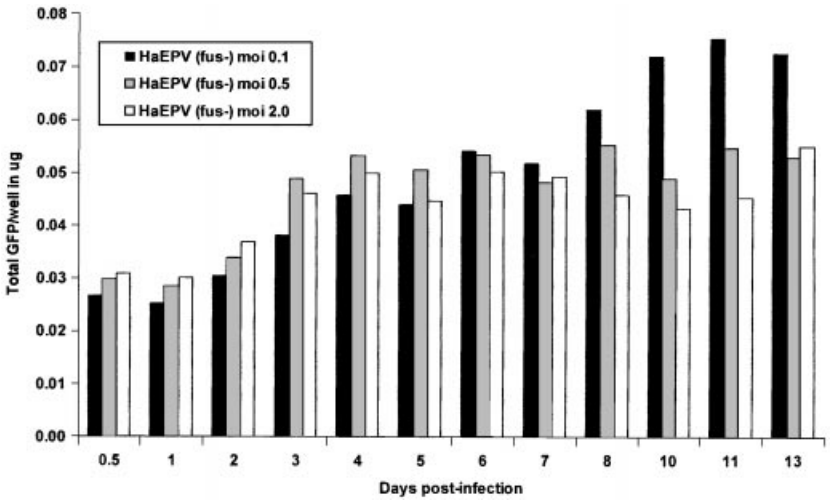


Fig. 5. Production of GFP in Sf9 cells infected with HaEPV(fus−). Data for production of GFP from HaEPV(fus−)-infected Sf9 cells, as shown in Fig. 4, are graphed to facilitate interpretation of synthesis kinetics. HaEPV(fus−) infections were initiated at an m.o.i. of 0·1 (black bars), 0·5 (grey bars) or 2·0 (open bars).

activity in this system, rather than some interference in viral replicative process(es).

## Discussion

Work reported here demonstrates that HaEPV is amenable to genetic manipulation, and that both the *sph* and *fus* loci can be used for this purpose. We have shown that neither the kinetics nor levels of ECV production are significantly altered by replacement of the native *sph* or *fus* coding sequences with one encoding a *gfp*(RS) marker protein, and on this basis we consider it unlikely that other parameters associated with intracellular virus replication were affected by the manipulations. We employed a 'seamless' strategy to generate recombinants of HaEPV. This approach allowed all non-coding native sequences to remain unaltered, and hence minimized the risk of inadvertent introduction of modifications with artefactual consequences for transcription, as has been described elsewhere (King *et al*., 1998). On the basis of these strategies and assessments, we consider that the estimates of marker protein synthesis presented here reflect genuine patterns of HaEPV promoter activity within the *in vitro* system utilized.

In order to assess whether HaEPV might be as useful for heterologous protein expression as established baculovirus-based systems, we used Sf9 cells to make direct comparisons of production of GFP by the two recombinant HaEPVs and a commercial 'BacPAK'-based baculovirus, which expressed the same protein from the *polh* promoter. In all instances we observed that the kinetics and amounts of protein produced were dependent on the m.o.i. used to establish infection. Thus, for all three m.o.i. values tested, GFP production by BacPAK-GFP(RS) occurred in a rapid burst between 2 and 5 days p.i. (Fig. 4); at m.o.i. values of 2·0 and 0·5, total GFP did not increase after 5 days p.i., while when an m.o.i. of 0·1 was used, maximum accumulation was reached at 7–8 days p.i. In contrast, GFP production by HaEPV(sph−) was always more gradual, and protein continued to accumulate over a period of 3–11 days p.i. (Fig. 4). Under optimal conditions for GFP production (m.o.i. of 0·1), the HaEPV(sph−) recombinant produced about 60% of the quantity synthesized by the BacPAK-GFP(RS) recombinant; under less favourable conditions (e.g. at an m.o.i. of 2·0), its comparative production was about 30%.

While our experiments show that HaEPV(sph−) virus produces less GFP than BacPAK-GFP(RS) in Sf9 cells, it is conceivable that these production kinetics may be favourable in some bioproduction situations. For example, an accepted difficulty of use of baculovirus systems for expression of some heterologous proteins, especially those which require extensive post-translational processing and/or secretion, is that the rapid course of baculovirus infection frequently compromises host cellular functions required for these purposes (Jarvis, 1997). Stably transformed insect cell lines have been developed which utilize early baculovirus or non-viral promoters to try to circumvent this problem (Jarvis, 1997; Hegedus *et al*., 1998). However, use of the strong *sph* promoter in the context of viable HaEPV-infected cells potentially presents another means by which this issue might be addressed, without the requirement for production of stable cell lines.

We consider the next major test of the utility of HaEPV as an expression system to be the production of protein(s) whose functionality requires post-translational modification and/or secretion; it will here be of interest to determine whether the slower kinetics of HaEPV infection result in production of better 'quality' products. It is also of considerable interest to assess the possibility of utilizing non-lytic recombinants of HaEPV for continuous production of protein, as opposed to the current 'batch' infections used for large scale recombinant protein production with baculovirus.

An unexpected result of this study was the observation that expression of GFP from the *fus* locus of HaEPV produced a considerably lower quantity of protein (3–7%) than expression

from the *sph* locus. Since fusolin is the major constituent protein of HaEPV spindle bodies (SB), and more abundant than spheroidin in viral preparations from *in vivo* infections (Lai-Fook & Dall, 2000; Dall *et al*., 1993) we expected that both loci would produce comparable levels of protein. Given that three pure isolates of HaEPV(fus−) showed similar levels of GFP expression (data not shown), we do not believe that the results reported here reflect some deficiency in the virus used in these studies. We do consider it likely that the HaEPV *fus* promoter is more active in some cell types than others, an hypothesis based in part on observed distributions of SB in tissue preparations from infected caterpillars (J. Lai-Fook & D. J. Dall, unpublished), and which is now amenable to testing via *in vivo* infection with these recombinants.

Although isolation of the recombinants described here proved to be a lengthy process, we have demonstrated that expression of GFP can be used to identify and select cells infected with recombinant forms of the virus. Other work in this laboratory (data not shown) has demonstrated that intergenic sequences of HaEPV can be used as sites for insertion and expression of heterologous coding sequences placed under control of HaEPV-derived promoters, and also that more than one such sequence can be expressed simultaneously. These latter findings offer a potential means to link effective expression of a desired product, as described here through use of the *sph* locus, with minimal expression of a selectable marker driven by another HaEPV promoter from an intergenic locus. Alternatively, a dual expression vector could be constructed to target the spheroidin locus, with the spheroidin promoter driving foreign gene expression in an opposite orientation with another promoter driving marker gene expression, as has been done with baculovirus expression systems at its polyhedrin locus (O'Reilly *et al*., 1992). Further development of transfer vectors and engineered parental viruses should provide more versatility and convenience, and lead to the refinement of a selectable HaEPV-based protein expression system.

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