Assembly of *Amsacta moorei* entomopoxvirus spheroidin into spheroids following synthesis in insect cells using a baculovirus vector

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The gene encoding the major occlusion body protein, spheroidin, of *Amsacta moorei* entomopoxvirus (AmEPV) was introduced into a baculovirus vector under control of the polyhedrin gene promoter. A recombinant virus produced large, ovoid occlusion body-like structures in both *Spodoptera frugiperda* and *Trichoplusia ni* cells. These structures resembled the spheroids found in AmEPV-infected *Lymantria dispar* cells, except they were devoid of virus particles and were not surrounded by a membrane- or envelope-like structure. These results were confirmed by immunofluoresence microscopy and Western blotting using a specific anti-peptide antibody to spheroidin, and suggest that the supramolecular assembly of spheroids is not dependent on other EPV-encoded gene products. Transmission electron microscopy and subcellular fractionation experiments revealed that the spheroid-like structures were assembled in both the nucleus and cytoplasm of the recombinant virus-infected cells. This contrasts with the solely cytoplasmic localization found in AmEPV-infected cells.

Entomopoxviruses (EPVs) are linear dsDNA viruses that infect insects. They were first described by Vago (1963) and have been isolated from several insect orders including Coleoptera, Lepidoptera, Orthoptera and Diptera (Arif, 1995). *Amsacta moorei* EPV is probably the most well characterized insect poxvirus, primarily because it replicates in cultured insect cells (Goodwin *et al.*, 1990; Marlow *et al.*, 1993; Winter *et al.*, 1995). Entomopoxviruses have a biphasic life-cycle producing both occluded and non-occluded virus particles. By analogy with insect baculoviruses, it has been proposed that the role of the occlusion body is to protect virions during horizontal transmission between insect hosts (Arif, 1995). The crystalline occlusion bodies (spheroids) form in the cytoplasm of infected cells and are composed primarily of the virus-encoded protein, spheroidin (114·8 kDa) (Bergoin *et al.*, 1970; Hall & Moyer, 1991). Three EPV spheroidin genes (*sph*) have been sequenced: from AmEPV (Hall & Moyer, 1991), *Melolontha melolontha* EPV (Sanz *et al.*, 1994) and *Choristoneura fumiferana* EPV (Li *et al.*, 1997). These studies have shown that the spheroidin genes are highly conserved between the three viruses with 97·2% similarity between AmEPV and CfEPV and 73·4% between CfEPV and MmEPV (Li *et al.*, 1997). Each *sph* coding region is characteristically preceded by an AT-rich region comprising the promoter including the TAAATG poxvirus late gene consensus sequence. Recent studies, in which the *sph* coding region was deleted from the virus genome, have shown that synthesis of spheroidin is non-essential for virus replication in cell culture (Palmer *et al.*, 1995). In addition, the coding region may be replaced by that of a heterologous gene to produce a vector for foreign gene expression (Palmer *et al.*, 1995).

A number of studies have examined the replication cycle of AmEPV in *Estigmene acrea* or *Lymantria dispar* (LD652) cells and have identified various stages in the morphogenesis and maturation of virus particles (Goodwin *et al.*, 1990, 1991; Marlow *et al.*, 1993). Three forms of mature virus particle have been identified: intracellular virus, extracellular virus and occluded virus. It has been suggested that the formation of mature spheroids requires the presence of virus particles or EPV-encoded proteins to initiate the occlusion process (Marlow *et al.*, 1993; Alaoui-Ismaili & Richardson, 1996). If this were correct, the formation of spheroids would differ from the supramolecular assembly of baculovirus polyhedrin, which can occur in the absence of virus particles. To further our understanding of the mechanisms involved in the formation of EPV spheroids, we expressed AmEPV *sph* using a recombinant baculovirus.

A 3 kbp DNA fragment encoding the *sph* coding region (Hall & Moyer, 1991) was cloned into the *Bam*HI site of the baculovirus transfer vector pAcAL1 (Lawrie *et al.*, 1995), under control of the polyhedrin gene promoter, to produce pAcSph. Restriction enzyme analysis and DNA sequencing was used to

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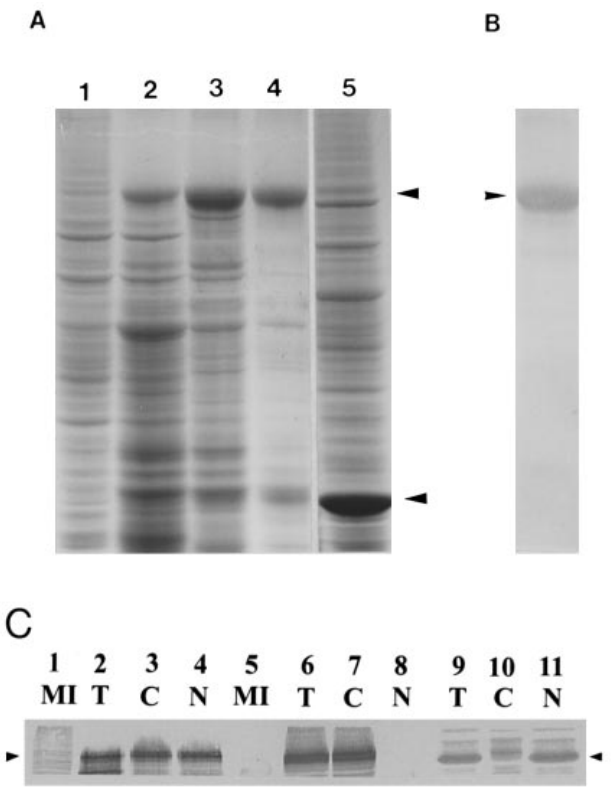

Fig. 1. Detection of recombinant spheroidin in TniHi5 cells. (A) TnHi5 cells in shake culture (Excell 401 medium) were infected with AcSph at a multiplicity of 10 p.f.u. per cell and incubated at 28 °C for 72 h. At 24 (lane 2), 48 (lane 3) and 72 (lane 4) h p.i., 1 ml samples of cells were pelleted by centrifugation, washed twice in PBS, resuspended in 25 µl PBS and disrupted by boiling in an equal volume of 2 × dissociation buffer, followed by electrophoresis through 10% SDS–polyacrylamide gels (King & Possee, 1992). The upper arrow indicates spheroidin (115 kDa). Mock-infected cells (lane 1) and wild-type AcMNPV cells (48 h p.i., lane 5) are also shown. The lower arrow indicates polyhedrin. (B) An immunoblot of lane 3 in (A) is shown (AcSph-infected cells at 48 h p.i.). Spheroidin is indicated by the arrow. Immunoblot analysis was carried out using standard procedures. Briefly, the blot was blocked in PBS containing 0·1% Tween 20 and 3% BSA and then incubated overnight at 4 °C in polyclonal anti-spheroidin antibody (diluted 1:500 in blocking buffer). The bound antibodies were detected using goat anti-rabbit IgG coupled horseradish peroxidase and DAB staining. (C) TnHi5 cells were infected with either AcSph (lanes 2–4) or wild-type AcMNPV (lanes 9–11) at 10 p.f.u. per cell. Mock-infected cells are shown in lane 1 (MI). As a control for the fractionation experiment, Ld652 cells were also infected with AmEPV at 10 p.f.u. per cell (lanes 6–8). Mock-infected Ld652 cells are shown in lane 5 (MI). At 48 h p.i. (AcMNPV and AcSph) or 120 h p.i. (AmEPV), nuclear and cytoplasmic fractions were prepared by cell lysis in the presence of 0·5% NP-40, followed by NaCl extraction, exactly as described by Bosher *et al.* (1992). Total cellular (lanes marked T), nuclear (lanes marked N) or cytoplasmic (lanes marked C) extracts were solubilized in dissociation buffer, separated by SDS–PAGE electrophoresis, and analysed by immunoblotting as previously described (King & Possee, 1992). Immunoblotting details are as described for (B), with the addition that a polyclonal antiserum specific for AcMNPV polyhedrin was used for the portion of the blot containing lanes 9–11.

confirm the correct orientation of the gene with respect to the polyhedrin gene promoter. *Spodoptera frugiperda* (IPLB-Sf-21AE; Sf21) cells were cotransfected with 200 ng *Bsu*36I-digested BacPAK6 virus DNA (Kitts & Possee, 1993) and 1000 ng pAcSph using Lipofectin (Gibco Life Technologies) as previously described (King & Possee, 1992). At 48 h post-transfection, the culture medium was harvested and putative recombinant viruses were isolated by plaque-assay. Following virus amplification in Sf21 cells, a recombinant virus was identified by dot-blot hybridization techniques (data not shown) and designated AcSph. This virus was amplified to high titre, 1×10^8 p.f.u./ml, in Sf9 cells grown in Sf900II serum-free medium (Life Technologies) in shake cultures. Preliminary experiments indicated that higher levels of spheroidin were produced in virus-infected TnHi5 cells (BTI-TN5B1–4) in Excell 401 medium (J.R.H. Bioscience), than in Sf9 cells (data not shown) and, therefore, the TnHi5 cells were used in all the subsequent studies.

A unique band of protein (~ 115 kDa) was observed in AcSph-infected TnHi5 cell extracts from 24 to 72 h p.i. (Fig. 1A, lanes 2–4, respectively). This protein was not observed in non-infected cells (lane 1) nor in wild-type AcMNPV-infected cells (lane 5). The novel protein was confirmed as spheroidin by Western blot analysis (Fig. 1B) using a spheroidin-specific anti-peptide polyclonal antiserum. The antibody was raised in rabbits using an N-terminal specific peptide sequence (MSN-VPLATKTIRKLSNRKC) conjugated to keyhole limpet haemocyanin.

Indirect immunofluorescence and phase contrast microscopy, performed as described in Marlow *et al.* (1992), indicated that spheroid-shaped, occlusion-like structures were formed in AcSph-infected TnHi5 (Fig. 2) and Sf9 cells (data not presented) at late times post-infection (p.i.). The synthesis of recombinant spheroidin monitored using anti-spheroidin antibody, and immunofluoresence microscopy, showed that the protein could be initially detected in a perinuclear distribution early in the infection (24 h p.i., Fig. 2A). By 48 h p.i., the distribution of the protein had become more cytoplasmic and globular in appearance (Fig. 2B). Later in the infection (72–96 h p.i.), spheroid-shaped bodies were visible by phase contrast microscopy (Fig. 2C), and were recognized by the anti-spheroidin antiserum (Fig. 2D). The morphology of these inclusion bodies closely resembled those observed in AmEPV-infected Ld652 cells by both immunofluoresence (Fig. 2E) and by phase contrast (Fig. 2F).

The intracellular distribution of the occlusion-like bodies was studied in more detail using cell fractionation followed by SDS–PAGE and immunoblotting. Virus-infected TnHi5 cells were harvested at 72 h p.i., and nuclear and cytoplasmic extracts (Bosher *et al.*, 1992) were analysed by electrophoresis through 10% SDS–polyacrylamide gels, followed by Western blotting with spheroidin-specific antiserum. As shown in Fig. 1(C), spheroidin was detected in the total cell extract (T), and in both the cytoplasmic (C) and nuclear (N) fraction of the recombinant AcSph-infected cells (lanes 2–4, respectively). In contrast, spheroidin protein was detected only in the total extract and cytoplasmic fraction of AmEPV-infected Ld652 cells (lanes 6 and 7, respectively). This was to be expected as

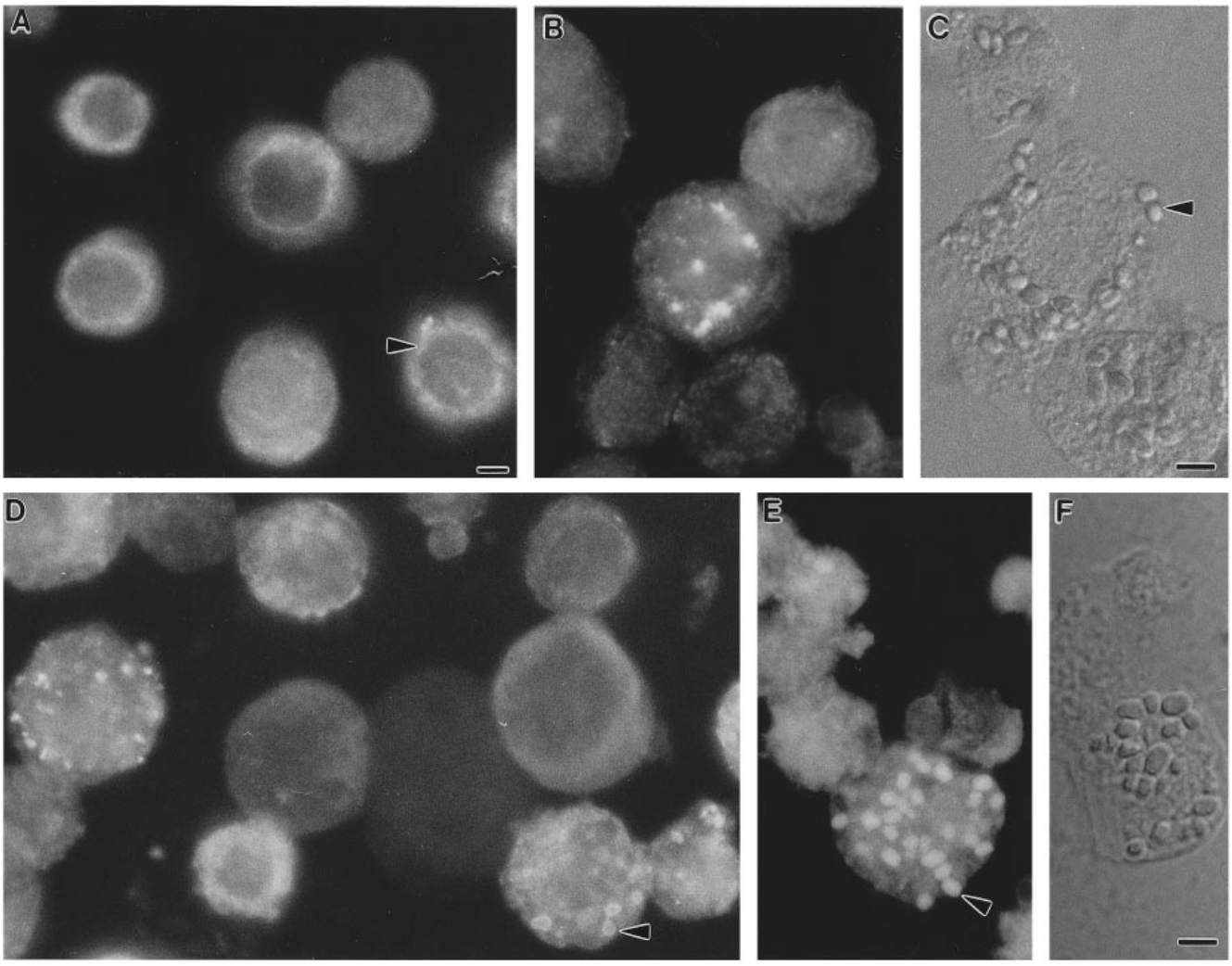

Fig. 2. Analysis of AcSph-infected cells by indirect immunofluorescence and phase contrast microscopy. TnHi5 cells were infected with AcSph at a multiplicity of 10 p.f.u. per cell. Cells are shown after 24 (A), 48 (B) and 72 (C and D) h p.i. Also shown are Ld652 cells infected with AmEPV at 96 h p.i. (E and F). Panels (C) and (F) show developing spheroids in virus-infected cells under phase-contrast microscopy and panels (A), (B), (D) and (E) show developing spheroids using indirect immunofluorescence microscopy. Typical spheroids or spheroid-like structures are indicated by arrows. For indirect immunofluorescence, TnHi5 and Ld652 cells were grown in 35 mm dishes containing sterile glass coverslips, and following virus infection for the times stated, the cell monolayers were washed in PBS, fixed and permeabilized in iced acetone for 15 min at −20 °C. After washing in PBS, the cells were incubated for 1 h at room temperature in primary antibody (anti-spheroidin diluted 1:500 in PBS containing 3% BSA). Antibody binding was detected using FITC-conjugated goat anti-rabbit IgG (Sigma), diluted 1:60 in PBS with 3% BSA. The cells were then washed three times in PBS (5 min each) and twice in distilled water, allowed to air dry and mounted in Citifluor (Agar Supplies). Bar, 2 μm.

entomopoxvirus infection occurs solely in the cytoplasm of infected cells (reviewed by Arif, 1995). As a control, wild-type *Autographa californica* nucleopolyhedrovirus (AcMNPV)-infected cells were fractionated and, as would be expected, the occlusion protein polyhedrin was detected only in the total extract (lane 9) and nuclear fraction (lane 11). Thus, the cell fractionation results indicated that when spheroidin was produced in recombinant baculovirus-infected cells, it was localized within both the nuclear and the cytoplasmic compartments.

These results were confirmed by examination of virus-infected cells by transmission electron microscopy (TEM) (Fig. 3). TnHi5 cells infected with recombinant AcSph or Ld652 cells infected with AmEPV were harvested and processed for TEM at various times p.i., as previously described (Marlow *et al.*, 1993). Native spheroids formed in Ld652 cells begin to occlude virus particles from 48 h p.i. (Fig. 3A), and to form mature spheroids between 96 and 120 h p.i. (Fig. 3B). The recombinant AcSph-infected cells show clearly that occlusion body-like structures were formed in both the nucleus and cytoplasm (Fig. 3C, D). These appeared to have a similar morphology and crystalline-like lattice structure, but lacked occluded virions

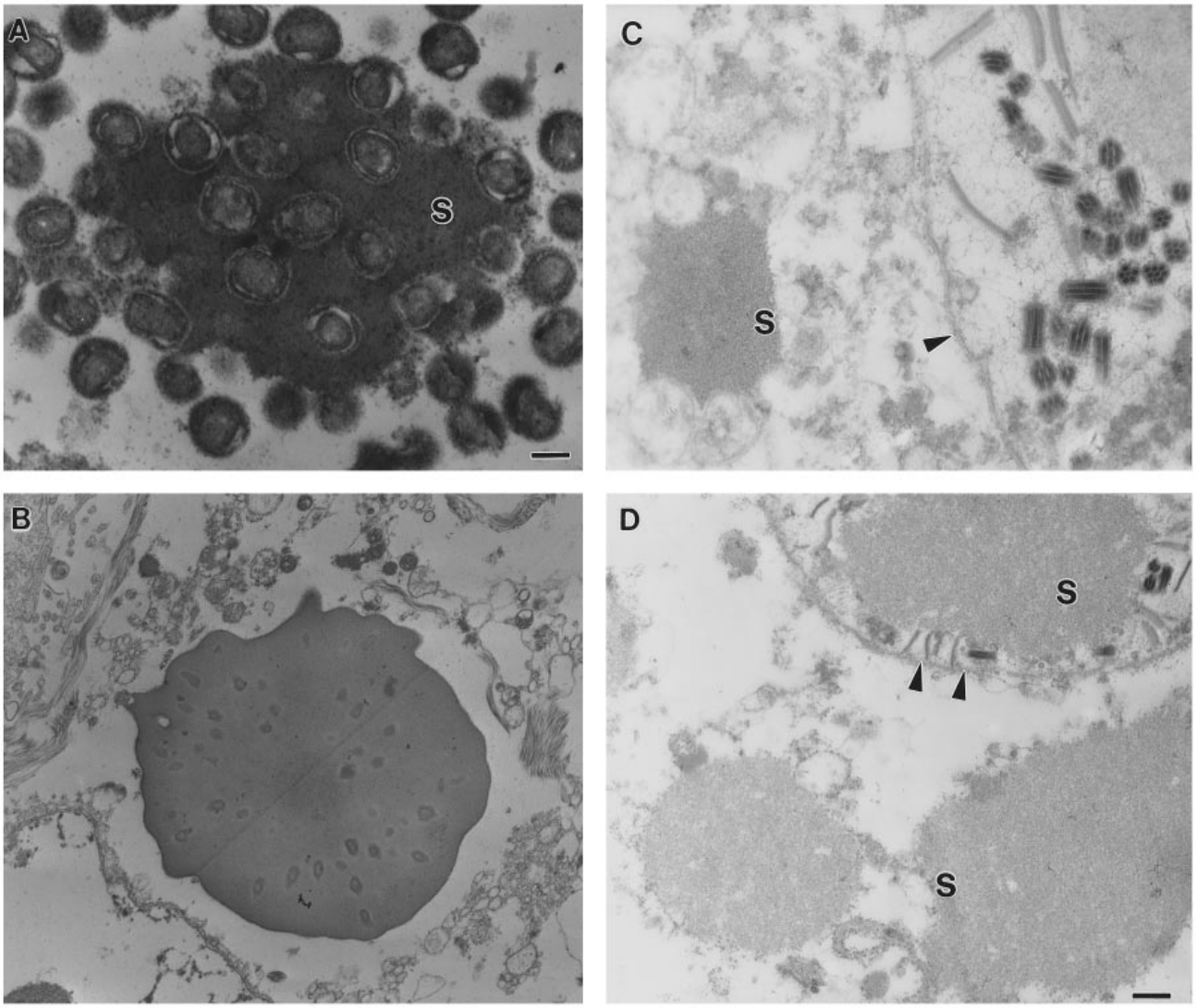

Fig. 3. Analysis of AcSph-infected cells by electron microscopy. AcSph-infected TnHi5 (C and D) and AmEPV-infected Ld652 cells (A and B) were harvested from monolayers at 48 (A and C) and 72 h p.i. (AcSph, D) or 120 h p.i. (AmEPV, B) by low speed centrifugation (2 min at 2000 r.p.m.), washed in PBS and resuspended in 1 % glutaraldehyde–1 % paraformaldehyde for 1 h at room temperature, followed by post-fixation in 0·1 % aqueous OsO_4. The cells were then dehydrated through an alcohol series, embedded in Spurr's resin and polymerized at 70 °C. Ultrathin sections (60–70 nm), mounted on 200 mesh copper grids were stained in 1 % uranyl acetate and lead citrate, washed five times (5 min each) in distilled water and air dried. In panels (A), (B) and (C) developing spheroids or spheroidin-like bodies are indicated (S) and the nuclear membrane is indicated by arrows in panels (C) and (D). Bar, 0·5 μm (A) and 0·2 μm (B–D).

and the envelope/membrane-like structure that is normally associated with mature spheroids (Fig. 3B). Spheroidin-like structures were first apparent at 48 h p.i (Fig. 3C) and were obvious throughout the cytoplasm and nucleus by 72 h p.i. (Fig. 3D). Interestingly, AcMNPV virions were not occluded into the spheroidin matrix formed in the nucleus of virus-infected cells (Fig. 3D). No occlusion-like structures were observed in control, BacPAK6-infected cells (data not shown).

The cell fractionation and microscopy data demonstrate that the AmEPV spheroidin protein has intrinsic properties that allow supramolecular assembly into spheroid-like occlusion bodies in the absence of other AmEPV gene products. In this respect, spheroid production appears to resemble the formation of polyhedra from polyhedrin in both the NPVs (reviewed by Rohrmann, 1986) and the cytoplasmic polyhedrosis viruses (CPVs) (Mori *et al.*, 1993). These results contradict earlier suggestions that EPV virus particles may be necessary to act as a nucleation point for the self-assembly of spheroidin into spheroids. However, the occlusion of virus particles to form mature spheroids is probably an active, multistep process that requires virions, and possibly other virus proteins, that are capable of recognizing spheroidin. In support of this conclusion, although NPV virions were observed to be close to the spheroid-like structures that

assembled in the nucleus of recombinant virus-infected cells, they were never observed to be occluded with the spheroidin matrix. Thus, the self-assembly of spheroidin and the occlusion of virus particles probably constitute distinct events in the formation of mature spheroids.

The localization of the spheroid-like occlusion bodies in both the cytoplasm and nucleus was not expected as in natural virus infections spheroids are confined to the cytoplasm (reviewed by Arif, 1995). Jarvis *et al*. (1991) reported that the amino acid sequence KRKK is important for the nuclear localization of the NPV polyhedrin. This sequence has similarities to the well characterized nuclear localization signals of other DNA viruses, including SV40 (PKKKRKV) (Kalderon *et al*., 1984; Lanford & Butel, 1984). Examination of the spheroidin sequence revealed no tract of basic amino acid residues similar to this signal. Our results were similar to those obtained following synthesis of a CPV polyhedrin gene using a recombinant baculovirus. The CPV polyhedrin assembled into cubic polyhedra in both the cytoplasm and nucleus; no explanation was given for this result (Mori *et al*., 1993). The reason that some of the spheroid-like structures assembled in the nucleus, therefore, remains unknown. One possibility is that spheroid formation utilizes the cellular cytoskeleton, particularly actin microfilaments, as an assembly framework. Our previous studies have shown that the cytoskeleton is rearranged extensively in AmEPV-infected cells (Marlow *et al*., 1992). In particular, f-actin is rearranged to form thick cables radiating throughout the cell (Marlow *et al*., 1992). Cudmore *et al*. (1995) have also shown that in vaccinia virus-infected cells, f-actin is rearranged to form projections or tails that help propel the intracellular enveloped virions to the cell surface, to contact and infect neighbouring cells. In baculovirus-infected cells it has been demonstrated that the actin component of the cytoskeleton is re-directed to the nucleus where it may form a framework upon which polyhedra are assembled (Charlton & Volkman, 1991, 1993). It could therefore be argued that if spheroidin uses a similar mechanism for its assembly, some protein may be fortuitously targeted to the nucleus during the concomitant relocalization of f-actin in baculovirus-infected cells. Studies are continuing to examine this hypothesis and also to investigate the domains necessary for the supramolecular assembly of AmEPV spheroidin.

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