

## A new ascovirus from *Spodoptera exigua* and its relatedness to the isolate from *Spodoptera frugiperda*

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A new ascovirus was isolated from *Spodoptera exigua* in Indonesia and was tentatively assigned as a new species, *Spodoptera exigua ascovirus 5a* (SeAV-5a) according to the present ICTV ascovirus naming scheme based on DNA restriction fragment length polymorphism (RFLP), hybridization, formation of occlusion body, tissue tropism and host spectrum. SeAV-5a replicated primarily in the fat body of susceptible hosts. SeAV-5a could be transmitted to *S. frugiperda*, *Pseudoplusia includens* and *Trichoplusia ni*, but not to *Heliothis virescens*. Infection with SeAV-5a arrested growth of the hosts, but prolonged their survival, which continued up to 33 days. Clusters of virions were seen inside the characteristic vesicles. Occasionally, virions were contained within vacuoles (one to five per vacuole) and some virions were embedded in occlusion bodies. The size of the SeAV-5a virion was 347 × 134 nm; however, aberrant long secondary viral products were also seen. The presence of occlusion body and Southern hybridization and Western immunoblot analyses suggest that SeAV-5a is more closely related to *S. frugiperda ascovirus 1a* (SfAV-1a) than to *Trichoplusia ni ascovirus 2* (TnAV-2). Certain regions of the 182 kb genome of SeAV-5a showed hybridization to that of SfAV-1a. Two fragments in each of the SfAV-1a *EcoRI* and *HindIII* digests hybridized to the SeAV-5a genomic DNA probe. Five to eight *HindIII* and *EcoRI* fragments in SeAV-5a DNA hybridized to the SfAV-1a genomic probe.

### Introduction

Ascoviruses are newly discovered viruses that infect lepidopteran species and have the unique feature of forming vesicles which accumulate in the haemolymph of infected hosts (Federici *et al.*, 1991). The vesicles, which contain virus particles, are formed by cleavage of infected host cells. Ascoviruses have large enveloped virions (130 × 400 nm), which are allantoid to bacilliform in shape, and have complex symmetry. Earlier reports on the configuration of their genomes indicated that they had a linear, double-stranded DNA of about 100–180 kb (Federici, 1983; Federici *et al.*, 1999). However, Bigot *et al.* (1997a) and Cheng *et al.* (1999) have reported that ascoviruses have a circular genome.

The first ascoviruses were isolated in 1977 from larvae of two different noctuid species, the cotton bollworm, *Heliothis*

*zea* (Adams *et al.*, 1979; Hudson & Carner, 1981; Carner & Hudson, 1983) and the clover cutworm, *Scotogramma trifolii* (Federici, 1982). Since then, ascoviruses have been isolated from larvae of *Autographa precationis* (Hamm *et al.*, 1986), *Heliothis virescens* (Hamm *et al.*, 1986), *Spodoptera frugiperda* (Hamm *et al.*, 1986) and *Trichoplusia ni* (Browning *et al.*, 1982). All these isolates were found within the continental United States. Recently, an ascovirus was isolated from larvae of the leek-moth, *Acrolepiopsis assectella*, in France (Bigot *et al.*, 1997b). Since the reported ascoviruses have a relatively wide host-range among noctuid species, ascoviruses are probably distributed worldwide (Federici *et al.*, 1991). A plausible reason for not having isolated ascoviruses until recently may lie in the pathology of the infected insect. The virus does not cause gross pathology and, therefore, infected insects are not easily identified in the field (Browning *et al.*, 1982; Hamm *et al.*, 1986; Federici *et al.*, 1990).

In this paper, we report the characterization of an ascovirus isolate from *Spodoptera exigua* in Indonesia, which represents the first isolate from Asia. Its relatedness to the previously reported *S. frugiperda ascovirus 1a* (SfAV-1a) is described.

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## Methods

■ **Insect rearing and virus propagation.** *S. exigua* and *S. frugiperda* larvae were kindly provided by John Hamm (ARS, Tifton, GA, USA). Larvae of *P. includens* and *H. virescens* were from colonies maintained in our laboratory. All larvae were reared on pinto bean-based artificial diet (Burton, 1969). The method of inoculation of larvae with SeAV-5a was basically the same as described by Govindarajan & Federici (1990). Briefly, third-instar *S. exigua* larvae were pierced at the base of a proleg with a fine insect pin (size 000) contaminated with SeAV-5a and then placed on artificial diet. At 7 days post-inoculation (p.i.), opaque white haemolymph was collected by piercing the base of a proleg of the diseased larvae. The haemolymph was stored at  $-20^{\circ}\text{C}$ .

The other ascoviruses used in these studies were SfAV-1a from *S. frugiperda* (a gift from B. A. Federici, University of California, Riverside, CA, USA) and TnAV-2 isolated from *H. virescens*. SfAV-1a was in the form of concentrated vesicles. TnAV-2 is actually a variant of TnAV-2a (Federici *et al.*, 1990). TnAV-2 was isolated from *H. virescens* from a cotton field in South Carolina. It was propagated in *H. virescens* as described above.

■ **Virus purification and DNA analysis.** Virus-containing vesicles from infected larvae were disrupted by ultrasonication and the virions were purified through sucrose density gradients (Federici *et al.*, 1990). Intact viral DNA was purified on CsCl-ethidium bromide gradients by ultracentrifugation.

Viral DNA was digested with restriction endonucleases (REN) (*Hind*III, *Eco*RI and *Pst*I) under the conditions recommended by the manufacturers (BioLabs or Promega) and analysed by 0.7% agarose gel electrophoresis with  $\lambda$ -*Hind*III DNA and 1 kb DNA ladder (GibcoBRL) as molecular size markers. The gel was stained with ethidium bromide and photographed with an IS-1000 Digital Imaging System (Alpha Innotech Corp., San Leandro, CA, USA). The accompanying software (version 2.02) was used to estimate the molecular mass of DNA fragments

■ **DNA hybridization analyses.** Genomic DNAs (500, 50 and 5 ng) from TnAV-2, SfAV-1a and SeAV-5a were digested with *Hind*III and *Eco*RI (Promega or BioLabs) and separated on 0.7% agarose gels. A 1 kb DNA ladder marker was used as a size marker and as a negative control in the following hybridization analyses. Fragments were blotted onto a Hybond N+ nylon membrane (Amersham) (Southern, 1975; Chomczynski, 1992). Genomic DNA probes (40 ng) of TnAV-2, SfAV-1a and SeAV-5a were digested with *Sau*3AI and the digested products (about 200 bp in length) were labelled with [ $^{32}\text{P}$ ]dATP with a random primer kit (GibcoBRL). Hybridization was carried out in  $6 \times \text{SSC}$ , 0.5% SDS and  $5 \times \text{Denhardt's}$  solution at  $65^{\circ}\text{C}$  for 21 h. The membrane was washed twice in  $2 \times \text{SSC}$  for 5 min at room temperature ( $23^{\circ}\text{C}$ ) and twice in  $2 \times \text{SSC}-0.1\%$  SDS for 30 min at  $65^{\circ}\text{C}$ .

■ **PAGE and immunological analyses.** Purified virions of each ascovirus were mixed with equal volumes of  $2 \times \text{SDS}$  sample buffer (containing the reducing agent 2-mercaptoethanol), boiled for 2 min and proteins separated on a 12% SDS-PAGE discontinuous gel. AcMNPV occlusion bodies were used as a protein size reference and a negative control in immunoblot analysis. Duplicate conditions of the above SDS-PAGE without staining were used for Western blot analysis. Separated proteins were transferred onto Nytran nitrocellulose membrane following the standard protocol for Western analysis. A 50 kDa (P50) protein common to all ascoviruses (Federici *et al.*, 1990) was cut out of the gel for antibody production. Antiserum was raised in rabbits by Cocalico Biologicals Inc. (Reamstown, PA, USA) and pre-immune serum was tested as a negative control. The P50 antiserum at a 1:5000 dilution was used to bind P50 on the membrane. Bonded antiserum was detected using a Promega ProtoBlot Western blot AP systems kit.

■ **Infectivity and host range of SeAV-5a.** The noctuid larvae tested for determining the host range of SeAV-5a were *P. includens*, *T. ni*, *H. virescens*, *S. frugiperda* and *S. exigua*. For each species, 22 to 60 third-instar larvae were injected with an insect pin contaminated with purified SeAV-5a as described above. For controls, pins were dipped in sterile water. Treated larvae were incubated on pinto bean-based artificial diet at  $27^{\circ}\text{C}$  and 70% relative humidity. Infection was ascertained by observing characteristic vesicles in the haemolymph as described above. Mortality was recorded daily until the treated larvae had either died or pupated. Effects of SeAV-5a infection on *S. exigua* and *S. frugiperda* were monitored by weighing infected larvae and control larvae individually until they pupated or died.

■ **Replication of SeAV-5a in Sf-21 cells.** The Sf-21 cell line was kindly provided by A. H. Wood (Boyce Thompson Institute, Cornell University, NY, USA). Cells were maintained as a monolayer in TNM-FH medium in  $25 \text{ cm}^2$  T-flasks at  $27^{\circ}\text{C}$ . Haemolymph was collected from the *S. exigua* larvae at 4 days p.i. by piercing a proleg. Cloudy white haemolymph was collected with a Pasteur pipette, suspended in 0.5 ml of TNM-FH medium and filtered through a  $0.45 \mu\text{m}$  filter. The filtrate was then inoculated into Sf-21 cells at 30% confluence. Cells were monitored daily and infection was confirmed by transmission electron microscopy (TEM).

■ **Electron microscopy.** For negative-stained TEM preparations, purified virions were stained with 2% phosphotungstic acid on a coated grid. Ultrastructure and histopathology were investigated as follows. Third-instar *S. exigua* were inoculated with SeAV-5a following the method described earlier. At 4 days p.i., haemolymph, fat body and tracheal epidermis were isolated and processed according to Granados & Lawler (1981). For examination of free vesicles, the haemolymph of infected larvae was harvested and centrifuged as described by Federici *et al.* (1990). The pellet was embedded and processed for electron microscopy.

## Results

### Viral DNA analysis

REN digestion generated a large number of fragments with each enzyme. Some of the restriction fragments had similar mobility, forming doublets or triplets, and resulting in a brighter ethidium bromide-stained DNA band than the band above it (Fig. 1). The restriction analysis revealed that the total size of the SeAV-5a genome was  $182.5 \pm 0.74 \text{ kb}$  (mean  $\pm$  standard error of mean).

Side-by-side comparative restriction profiles (*Hind*III and *Eco*RI) indicated that SeAV-5a was not the same isolate as SfAV-1a or TnAV-2 (Fig. 2A). Southern hybridization studies indicated that SeAV-5a shared cross-hybridization regions with SfAV-1a but not with TnAV-2a. These cross-hybridization regions between SfAV-1a and SeAV-5a were detected when 500 ng genomic DNA was loaded in each lane of the agarose gel electrophoresis. Cross-hybridization regions were not detectable in the 50 and 5 ng loading (Fig. 2B, C; data for 5 ng loading not shown). Southern blot hybridization showed that labelled genomic SeAV-5a DNA hybridized strongly to the SfAV-1a 17.9 and 10.7 kb *Hind*III fragments, and the 14 and 10.7 kb *Eco*RI fragments (Fig. 2B). Labelled SfAV-1a genomic DNA hybridized to five and eight fragments, respectively, from *Hind*III and *Eco*RI digestion of SeAV-5a (Fig.

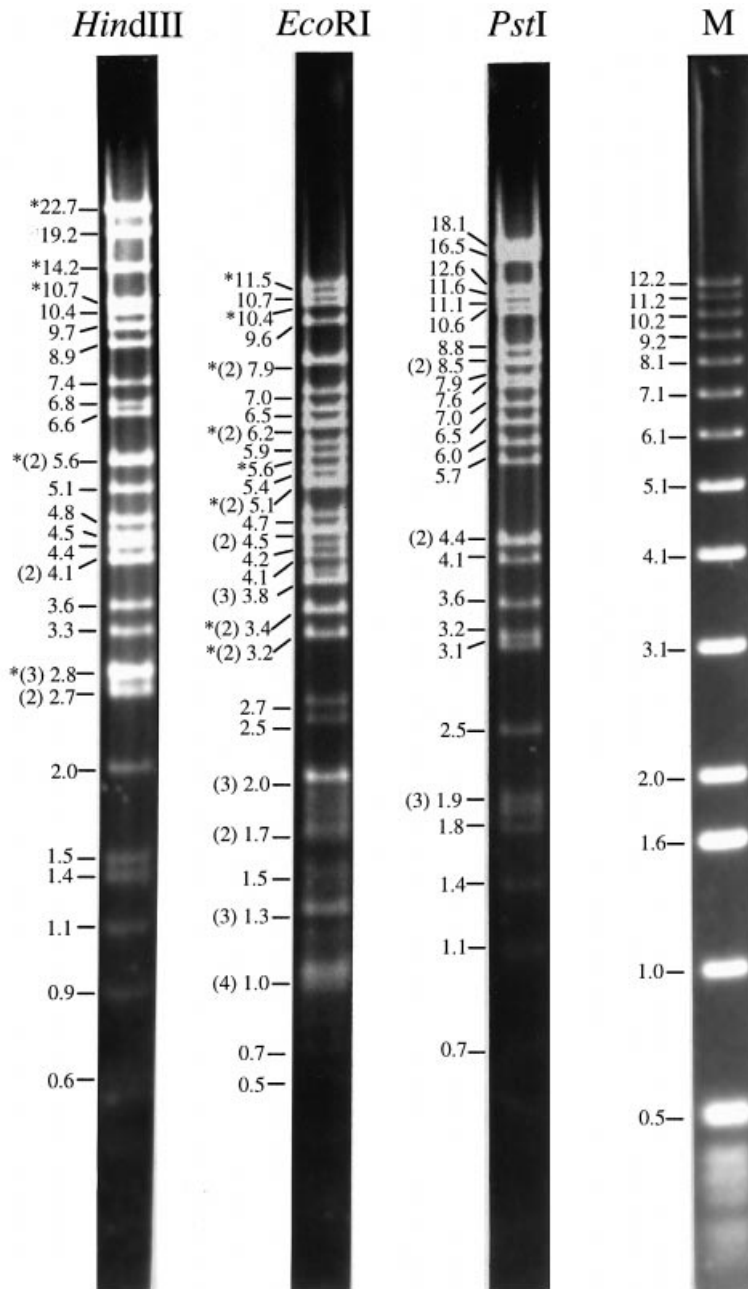


Fig. 1. Restriction endonuclease digestion of SeAV-5a DNA with *Hind*III, *Eco*RI and *Pst*I. Fragment size is in kbp. Numbers in parentheses are the number of fragments in the band indicated. Fragments of SeAV-5a marked with (\*) hybridized to the SfAV-1a genomic DNA probe. Lane M, size standard ( $\lambda$  1 kb DNA ladder).  $\lambda$  *Hind*III DNA (not shown) and 1 kb DNA ladder were used as molecular size markers.

2C). TnAV-2 probe hybridized to its homologous genomic DNA but not to either of the other two ascoviruses, SfAV-1a and SeAV-5a (Fig. 2D). These data suggest that there is conservation of certain regions or genes in the two ascovirus genomes.

#### Viral protein analysis

At least 22 SeAV-5a virion peptides were identified by discontinuous SDS-PAGE stained with Coomassie blue. Two highly expressed viral structural proteins (50 and 81 kDa) in

the SeAV-5a virions were more prominent than other proteins (Fig. 3A). The P50 was about 3 and 5 kDa smaller than the corresponding proteins in SfAV-1a and TnAV-2, respectively. SeAV-5a had another abundant protein (60.7 kDa) which seemed absent from SfAV-1a and TnAV-2. A 10.2 kDa protein was also abundantly expressed and common among the three ascoviruses (Fig. 3A). When rabbit antiserum raised against SeAV-5a P50 was used in immunoblotting tests to total virion proteins, it reacted strongly with the SeAV-5a P50, to a lesser extent with the SfAV-1a P53 and did not react at all with the TnAV-2 proteins (Fig. 3B). This suggests closer conservation

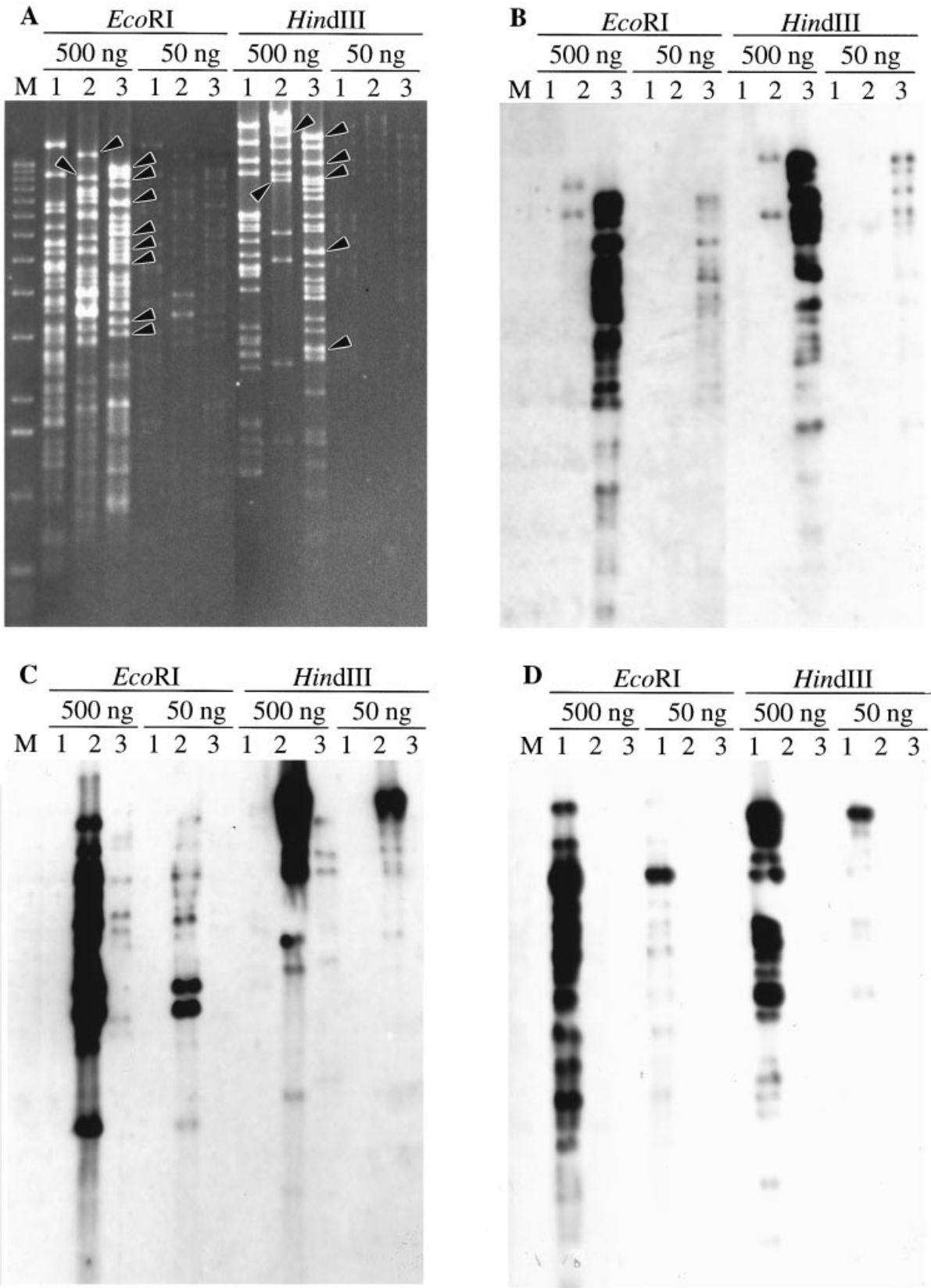
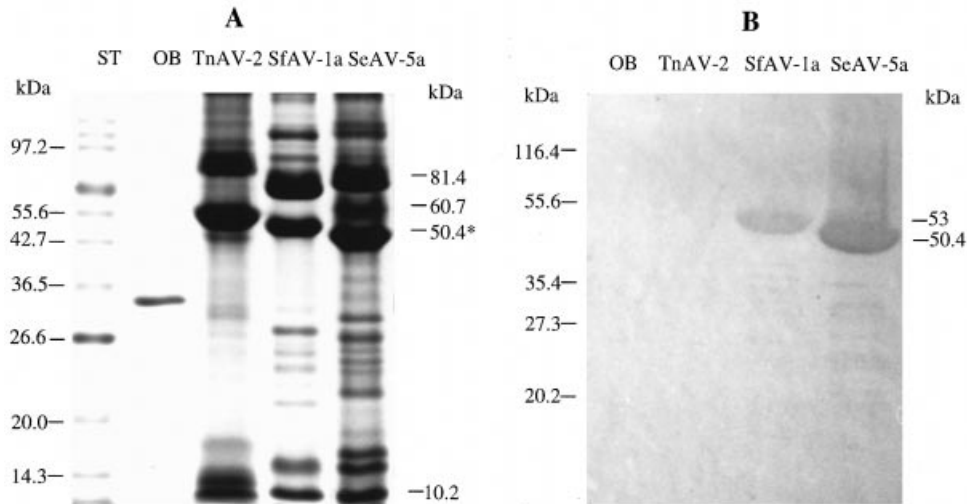


Fig. 2. Comparative restriction profile and Southern DNA hybridization analysis to establish the relatedness among TnAV-2 (lane 1), SfAV-1a (lane 2) and SeAV-5a (lane 3). (A) 500 or 50 ng of each DNA were digested with *HindIII* or *EcoRI*,



**Fig. 3.** Virion protein analysis of TnAV-2, SfAV-1a and SeAV-5a. (A) Comparison of protein composition of TnAV-2, SfAV-1a and SeAV-5a on a 12% SDS-PAGE gel stained with Coomassie blue. The numbers to the left indicate the molecular mass of protein standards (ST). Numbers to the right indicate the calculated sizes of the SeAV-5a proteins. P50 (marked \*) was used for antibody production in rabbits. OB, AcMNPV occlusion body as protein size reference. (B) Western blot analysis of TnAV-2, SfAV-1a and SeAV-5a. Proteins of TnAV-2, SfAV-1a and SeAV-5a virions were separated by 12% SDS-PAGE and blotted to nitrocellulose membranes. Antiserum against P50 of SeAV-5a was used to react with proteins on the blot. Visualization of antibody binding was achieved by a second anti-rabbit IgG AP-conjugated antibody. The numbers to the left indicate the protein molecular size standards.

**Table 1.** Host-range test of SeAV-5a against various lepidopteran hosts

Controls are not listed in the table, but all insects pupated at 4–5 days p.i. Numbers in parentheses represent the range (days p.i.) at which mortality occurred.

Species	No. tested	Mortality (%) at various stages			
		Larva	Prepupa	Pupa	Adult
<i>P. includens</i>	60	28.3 (7–12)	46.7	0	25
<i>T. ni</i>	22	45.4 (6–24)	13.6	41.0	0
<i>S. frugiperda</i>	60	86.2 (12–26)	0	13.8	0
<i>S. exigua</i>	60	100.0 (7–33)	0	0	0
<i>H. virescens</i>	60	0	0	0	0

of antigenic sites between the homologous protein of SeAV-5a and SfAV-1a.

### Infectivity of SeAV-5a

SeAV-5a had a profound effect on the growth and development of the host from which it was first isolated and on some other heterologous hosts. When *S. exigua* larvae were injected with haemolymph of SeAV-5a-infected larvae, typical ascovirus vesicle formation was observed at 2 days p.i. The haemolymph became milky white by 3 days p.i. The SeAV-5a-

infected larvae stopped gaining weight by 3 days p.i. but continued to feed and to lose weight until death between 7 and 33 days p.i. Control larvae developed normally and pupated in about 4 days (Table 1 and Fig. 4). Early death occurred when some larvae experienced moulting difficulty. In this case, the larvae could not cast the moulted exuvium completely, resulting in a partial moult and eventual larval death. A similar pathology was observed when *S. frugiperda* was infected with SeAV-5a (Table 1 and Fig. 4).

SeAV-5a was infectious to other lepidopteran hosts including *P. includens*, *T. ni* and *S. frugiperda*, but not to *H.*

separated on a 0.7% agarose gel and blotted onto filters for hybridization at 65 °C for 21 h. Arrowheads point to the hybridized fragments in the reciprocal hybridization analyses. (B) Blot was exposed to labelled SeAV-5a genomic DNA probe. (C) Blot was exposed to labelled SfAV-1a genomic DNA probe. (D) Blot was exposed to labelled TnAV-2 genomic DNA probe.

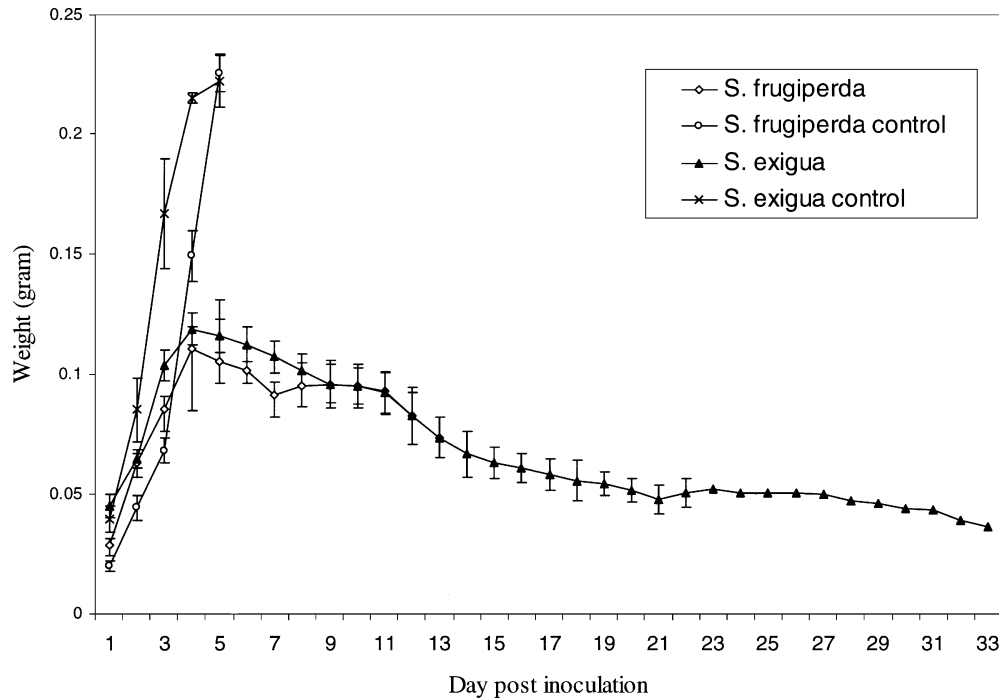


Fig. 4. Weight changes of *S. exigua* and *S. frugiperda* larvae after inoculation with SeAV-5a. Thirty larvae were initially used for the test and the indicated weights are the average of the living larvae. Vertical bars represent the standard error of mean ( $n = 1-30$ ).

*virescens*. All inoculated *H. virescens* larvae pupated and subsequently emerged as healthy adults.

The response of lepidopteran larvae to infection by SeAV-5a was varied. In the case of *S. exigua*, larvae were unable to develop into healthy adults and most died in the larval stage. Approximately 71.7% of the *P. includens* larvae grew to the prepupal stage and a portion (46.7%) of them died in the pupation cocoon. The rest (25%) developed into pupae that were smaller than the healthy ones. Adults that emerged from these smaller pupae showed deformed wings that would not spread out. Most infected *S. frugiperda* larvae (86.2%) died in the larval stage. Some larvae (13.8%) survived to the pupal stage, but emergence did not occur (Table 1).

The cell line Sf-21 was found to be permissive to SeAV-5a and infection was manifested by early deformation of the cell contour at 2 days p.i. Bulbous protrusions from infected cells formed at 3 days p.i., and the cells eventually lysed and released refractive vesicles (Fig. 5A). We observed that infection was best when inoculation was made at 30% cell confluence. If cells were inoculated at above 60% confluence, they became overgrown, making it difficult to see infection. Progeny virus (100  $\mu$ l) harvested from the medium at 5 days p.i. was infectious to healthy Sf-21 cells.

#### Electron microscopic investigation of SeAV-5a

Negative staining of SeAV-5a revealed that the virion shape varied from ovoidal to bacilliform (Fig. 5B). The size of

the virion was about  $347 \pm 13$  nm in length and  $134 \pm 21$  nm in width (60 counts). The surface of the viral envelope exhibited a reticulate pattern typical of that seen in other ascoviruses (Fig. 5B insert).

Ultrathin sections of the tissues of *S. exigua* infected by SeAV-5a revealed that this ascovirus replicated primarily in fat body cells, not in other tissues examined. Initial replication of SeAV-5a in the nuclei was characterized by the formation of a virogenic stroma and a concomitant deformation of the nuclear and cell membranes. The cell membrane also assumed an irregular shape as the cell became deformed. Some mature virions were observed in the cytoplasm of the infected fat body cells (Fig. 5C). Normally, the virions were contained in vesicles (Fig. 5D), but sometimes virions were observed within vacuoles in the vesicles (Fig. 5E). The number of virions in the vacuoles varied from one to five and some vesicles contained empty vacuoles (Fig. 5F, G). When fat body tissue was examined over time, no relationship could be established between the number of vacuoles and time p.i. In the process of virion purification, the haemolymph was sonicated and centrifuged. The pellet was found to be composed entirely of virions within vacuoles. When these vacuoles were analysed by SDS-PAGE, they exhibited the same peptide pattern as purified virions (data not shown).

SeAV-5a vesicles from *S. exigua* measured about  $5 \times 4$   $\mu$ m, were generally oval shaped and were packed with virions (Fig. 5D, F). The virions formed clusters in the vesicles and

occasionally a big vacuole not containing virions could be seen (Fig. 5F, G). Mitochondria were also enclosed in the vesicles and located at the periphery (Fig. 5D). Vesicles in the infected fat body tissues had rough surfaces while the free vesicles in the haemolymph had a relatively smooth appearance (Fig. 5D, F). Aberrant long secondary viral products (about 1.6 µm) were observed in the vesicles (Fig. 5G) and in some cases the virions were embedded in occlusion bodies (Fig. 5G, H). The size of the occlusion body was about  $1 \times 1.5$  µm.

## Discussion

Since the *Ascoviridae* is a family only recently recognized by the International Committee on Taxonomy of Viruses (ICTV), the species concept is still being developed and is subject to change as more reported ascovirus isolates are investigated in more detail (Federici *et al.*, 1999). At present, the following characters are used in combination to differentiate species in the genus: virion morphology, presence of occlusion body, lack of DNA–DNA hybridization with other species at low stringency, restriction enzyme fragment length polymorphism (RFLP), host of isolation and experimental host range, association with specific hymenopteran parasites and DNA polymerase sequence, if available (Federici *et al.*, 1999). Based on the above criteria, four ascovirus species have at present been recognized by the ICTV. These include *Diadromus pulchellus ascovirus 4a* (DpAV-4a), *Heliothis virescens ascovirus 3a* (HvAV-3a), *Spodoptera frugiperda ascovirus 1a* (SfAV-1a) and *Trichoplusia ni ascovirus 2a* (TnAV-2a). The number after the viral species indicates the chronological order of discovery. The lower-case letter following the number represents the genotype based on RFLP that is recognized for the accepted species (Federici *et al.*, 1999).

These four accepted ascovirus species could not be cross-hybridized at either high or low stringency conditions. SfAV-1a and HvAV-3a replicate primarily in the fat body tissue of susceptible hosts. TnAV-2a has a wide tissue tropism, replicating in several tissues including the fat body, tracheal matrix and epidermis of infected larvae. DpAV-4a replicates primarily in the pupal stage, where fat body and midgut are the primary tissues attacked (Bigot *et al.*, 1997b). TnAV-2a and HvAV-3a have a wide experimental host-range among larvae of the Noctuidae. However, the SfAV-1a host-range is restricted primarily to species of *Spodoptera*, and DpAV-4a infects species of the family Yponomeutidae. SfAV-1a virions are bacilliform and can be found occluded in vesiculate occlusion bodies, whereas TnAV-2a virions are allantoid and are not occluded in occlusion bodies. HvAV-3a virions are ovoidal to bacilliform, and are not occluded in occlusion bodies (Federici *et al.*, 1999).

SeAV-5a could not hybridize to TnAV-2, but some regions of the genome hybridized to SfAV-1a (Fig. 2B). Hybridization of SeAV-5a to HvAV-3a and DpAV-4a was not performed

because of the unavailability of the viruses. It is unlikely that this new ascovirus from *S. exigua* is the same as DpAV-4a because it has a much larger genome. Also, host range and pathology are quite different between SeAV-5a and DpAV-4a (Bigot *et al.*, 1997a, b). In addition to the partial hybridization to SfAV-1a, SeAV-5a formed an occlusion body structure similar to that of SfAV-1a but with a different matrix structure (Fig. 5D, G, H). The occlusion body of SfAV-1a has a vesiculate structure, but the occlusion body of SeAV-5a resembles that of a baculovirus polyhedron, which lacks the vesiculate structure (Federici *et al.*, 1990; Fig. 5G, H). SeAV-5a has a wider host range than SfAV-1a, which is restricted to the genus *Spodoptera* (Hamm *et al.*, 1986; Table 1). The wide host range of SeAV-5a resembles that of HvAV-3a, but the former does not infect *H. virescens* larvae and the latter does not form occlusion bodies (Table 1). Thus, the ascovirus isolate from *S. exigua* could not be assigned to any of the four reported ascoviruses in the present classification scheme and therefore has been tentatively assigned a new species code (SeAV-5a) in this report.

Since Southern and Western analysis (Fig. 2B, C, Fig. 3B) showed some relatedness between SeAV-5a and SfAV-1a, SeAV-5a may be related more closely to SfAV-1a than to TnAV-2. However, previous reports of dot blot and Southern hybridization did not establish this relatedness between SfAV-1a and other ascoviruses (Federici *et al.*, 1990; Hamm *et al.*, 1998). It is still not known how closely SeAV-5a and SfAV-1a are related, since genome organization and gene sequences are not well characterized. At present, only the DNA polymerase gene sequence of SfAV-1a has been published (Pellock *et al.*, 1996). In Southern blot analysis, particular REN fragments of SfAV-1a hybridized to SeAV-5a DNA and vice versa (Fig. 2B, C). This suggests that certain region(s) or gene(s) may be conserved between the two viruses. It is conceivable that the genes encoding P50 hybridized with each other since the homologous proteins have conserved antigenic sites (Fig. 3B). Unless the two hybridizing fragments in each digest are contiguous on the genome, it appears that there are other regions of high homology between SfAV-1a and SeAV-5a (Fig. 2B, C).

It is curious that some SeAV-5a virions were embedded in the occlusion body structure within the vesicle (Fig. 5D, G, H). Some were also found embedded in a similar structure at the periphery of the vesicles (Fig. 5D). The function of these occlusion bodies may be similar to that of baculoviruses, namely to protect the virus from adverse conditions outside the host (Federici *et al.*, 1990).

A novel structure consisting of vacuoles enclosing several virions was observed in the vesicles (Fig. 5E). This structure was resistant to the ultrasonication employed in breaking the vesicles to release virions. SDS–PAGE analysis of the protein composition of this structure did not show any additional proteins compared with those found in virions, which may indicate that they do not contain protein (data not shown). The

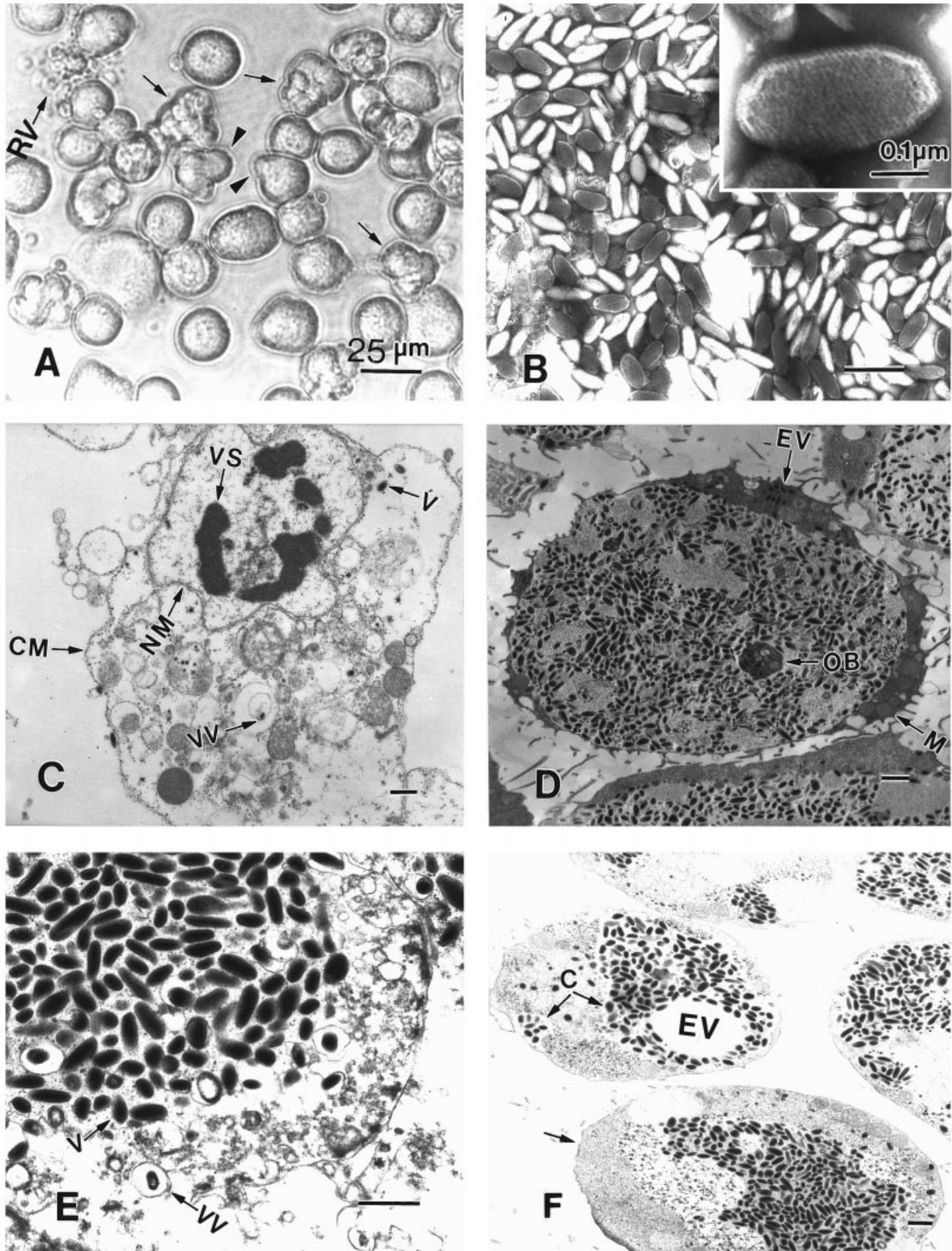
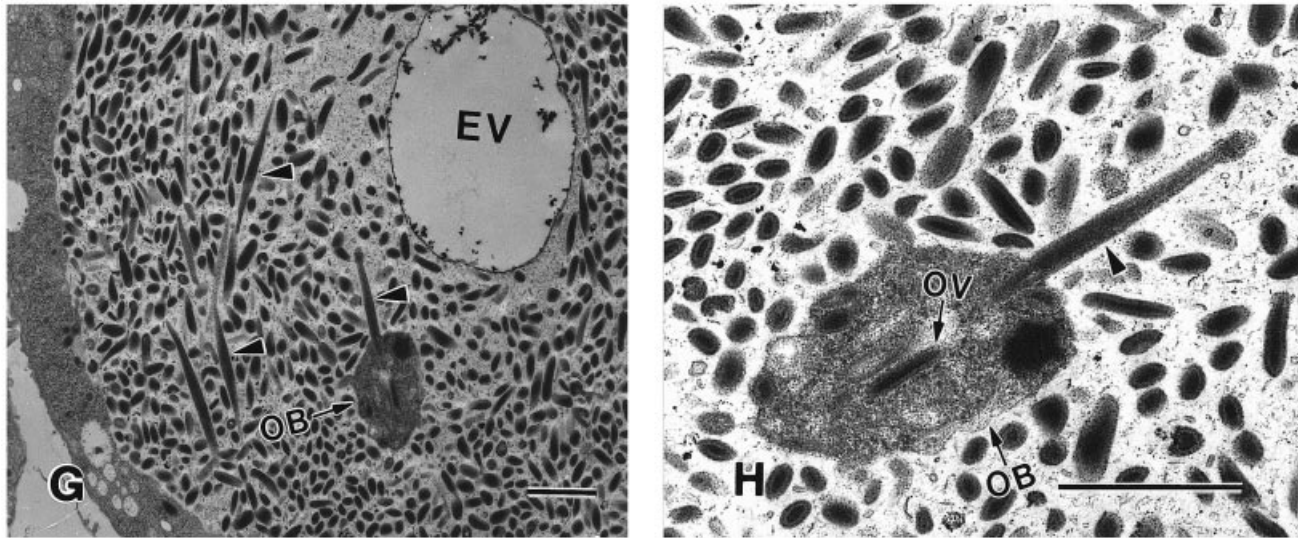


Fig. 5. For legend see facing page.



**Fig. 5.** Microscopic investigation of SeAV-5a infection. Bar markers represent 500 nm unless otherwise indicated on the bar marker. (A) Sf-21 cells 3 days p.i. with SeAV-5a. Arrowheads indicate the beginning of cell deformation caused by SeAV-5a. Arrows point to the changes observed at the later stages of cell infection. RV, released vesicles. (B) Negatively stained purified SeAV-5a virions under TEM. Insert shows the enlargement of one virion displaying the reticulated virion surface structure. (C) A cell of an *S. exigua* larva showing the virogenic stroma (VS) in the nuclei at 3 days p.i. with SeAV-5a. NM, nuclear membrane; CM, cytoplasmic membrane; V, virion. (D) A virion-containing vesicle in the fat body of an *S. exigua* larva. OB, occlusion body; EV, embedded virions; M, mitochondrion. (E) A virion-containing vesicle in the fat body of an *S. exigua* larva infected by SeAV-5a, showing the different types of virions. V, conventional virion; VV, virion in vacuoles. (F) Vesicles of SeAV-5a in the haemolymph of an *S. exigua* larva, showing the smooth surface of the vesicle (arrow). C, clusters of virions; EV, empty vacuole. (G) SeAV-5a vesicle from *S. exigua* showing long virions or secondary viral products (arrowheads), an occlusion body (OB) and an empty vacuole (EV) within the vesicle. (H) Enlargement of the occlusion body (OB) from (G) to show the detail of the occlusion body structure and a long virion or secondary viral product (arrowhead). OV, occluded virion.

vacuoles may be formed by invagination of plasma membrane of the infected cells. The function of this structure is unknown but it may protect the virions in the environment.

The effects of ascoviruses on the development and growth of infected hosts have been documented for other isolates (Govindarajan & Federici, 1990; Carner & Hudson, 1983; Hamm *et al.*, 1986; Federici & Govindarajan, 1990). SeAV-5a infection arrested development in all susceptible hosts (Fig. 4, Table 1). The response of the various insect species to SeAV-5a was reflected by changes in the appearance of the haemolymph. In all susceptible species (*S. exigua*, *S. frugiperda*, *T. ni* and *P. includens*) the haemolymph turned milky white. In the future, it may be useful to correlate haemolymph whiteness with level of virus production. The mechanism of larval growth retardation by ascoviruses has not been elucidated, but bears marked resemblance to that caused by polydnviruses. The latter group of viruses appears to reduce synthesis of ecdysone in the prothoracic gland (PTG), and consequently extends the larval life span (Dover *et al.*, 1988). Whether ascoviruses have the same effect on the PTG remains to be shown.

Infection of SeAV-5a *in vitro* to cell line Sf-21 should provide a vehicle to study this group at the cellular level. The effect of SeAV-5a on Sf-21 is similar to that of SfAV-1a (Federici & Vlask, 1986). Techniques for transfection of cells

with viral DNA need to be developed to allow production of recombinant viruses for gene expression studies.

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