

A novel small RNA virus isolated from the cotton bollworm, *Helicoverpa armigera*

Terry N. Hanzlik,* Susan J. Dorrian, Karl H. J. Gordon and Peter D. Christian

CSIRO Division of Entomology, Box 1700, Canberra, ACT 2601, Australia

A small RNA virus with novel characteristics has been isolated from laboratory-bred larvae of *Helicoverpa armigera*. Infection by the *H. armigera* stunt virus causes severe retardation of larval development and subsequent death. Its particles are isometric, 38 nm in diameter, and have a buoyant density of 1.296 g/ml in caesium chloride. The viral capsid has two major non-glycosylated protein components with M_s of 65 000 and 6000, and contains a genome composed of two non-polyadenylated single-stranded RNA molecules with lengths of 2.4 kb and 5.5 kb. The 5' termini of these RNAs are capped; their

3' termini are unblocked. *In vitro* translations of the viral RNAs showed synthesis of large proteins of sizes near the maximum coding capacity of each strand along with synthesis of numerous smaller proteins; no evidence for processing of precursors was seen. The physicochemical properties of the virus are most similar to those of the Nudaurelia ω virus, a provisional member of the Tetraviridae, although no antigenic relationship was observed between the two viruses. The bipartite genome and distinct capsid structure of these two viruses indicate the existence of a previously unrecognized virus group.

Introduction

Viruses pathogenic to heliothine moths are of interest to those concerned with agriculture as well as to virologists. The potential exists for these pathogens to be used as biological control agents against this group of moths, which includes a number of species that are economically important agricultural pests. Most reports on viral pathogens that infect the Heliiothinae describe viruses with large enveloped particles that contain double-stranded genomes, such as baculoviruses and reoviruses. In contrast, there are only two reports in the literature of small, non-enveloped, single-stranded RNA viruses infecting these insects. Scotti *et al.* (1981) reported an isolate of the picornavirus, cricket paralysis virus, that infects *Helicoverpa zea* (formerly *Heliothis zea*) and Rubinstein (1979) presented brief details of a small RNA virus isolated from larvae of *Helicoverpa armigera* that were also infected by a baculovirus. Although not sufficient to classify the virus, the details presented suggested the virus was also picorna-like.

During analysis of stunted or dead insects isolated from our colony of *H. armigera*, bands from caesium chloride gradients were found to contain particles that showed characteristics typical of a small RNA virus. When these particles were fed to neonate larvae of *H. armigera*, the larvae became lethargic within 1 day, failed to gain weight, ceased development and died in 3 days. Infection of third instar larvae caused a similar pattern with cessation of growth and feeding after 2 days and

death approximately 8 days post-infection. Examination of thin sections by transmission electron microscopy of severely stunted larvae showed that their midguts were largely disintegrated and heavily infested with the isometric virus particles. This paper reports physicochemical and biochemical characteristics of the particles of this small RNA virus which we have named *H. armigera* stunt virus (HaSV). Manuscripts describing the gross pathology (T. N. Hanzlik, S. Dorrian & P. Christian, unpublished) and the ultrastructural changes (K. C. Binnington, P. Christian & E. Brooks, unpublished) caused by this virus are in preparation.

Methods

Animals and virus production. *H. armigera* larvae were raised as described by Teakle & Jensen (1985). Virus production was in third instar larvae fed 10 mg pieces of diet to which 0.064 A_{260} units HaSV had been added. The larvae were grown for 8 days and were then collected and frozen at -80°C until further use. Frozen larvae (100 g) were homogenized in 200 ml of 50 mM-Tris-HCl buffer pH 7.4, filtered, and centrifuged in a Sorvall SS-34 rotor at 10000 g for 30 min. The supernatant was recentrifuged through a 10% sucrose cushion in a Beckman SW-28 rotor at 100000 g for 3 h and the pelleted virus was suspended overnight in 1 ml of buffer at 4°C . The resuspended virus was layered into a Beckman SW-41 tube containing equal volumes of 60% and 30% CsCl (w/v) and centrifuged for 12 h in a SW-41 rotor at 200000 g . The light-scattering, opalescent band of virus particles was removed from the gradient and then pelleted at 100000 g for 3 h in a SW-28 rotor in buffer. The pellet was suspended in 100 μl of buffer and frozen until further use.

Particle characterization. Staining with acridine orange was as described in Mayor & Hill (1961). Buoyant density was estimated in

CsCl gradients according to Scotti (1985). The accuracy of this technique was verified by obtaining a buoyant density of 1.36 g/ml for cricket paralysis virus in parallel experiments (Moore, 1991*b*).

Immunological procedures. Rabbit antiserum against HaSV particles was produced by standard immunological procedures. Rabbit antiserum against particles of the Nudaurelia ω virus (N ω V) and the virus itself were kindly provided by Don Hendry (Rhodes University, Grahamstown, South Africa). Rabbit antiserum against particles of the Nudaurelia β virus (N β V) was supplied by the late Carl Reingaum (Plant Research Institute, Burnley, Victoria, Australia). The serological relationship of HaSV to N ω V was determined by the standard reciprocal double diffusion technique as described by Mansi (1958). Immunoblotting was performed according to Towbin *et al.* (1979). Antibodies monospecific for the major 65K capsid protein of HaSV were prepared by incubating the rabbit polyclonal antiserum with sections of nitrocellulose that had been blotted with the 65K protein. After washing in buffer containing 150 mM-NaCl, the bound antibodies were eluted in 50 mM-citric acid buffer pH 2.5, and neutralized with 1.0 M-Tris-HCl pH 8.0 after a 5 min incubation at room temperature.

Protein characterization. SDS-PAGE followed the procedure of Laemmli (1970) and used 12.5% gels with low M_r standards (Bio-Rad) unless otherwise noted. Gels were stained with a colloidal preparation of Coomassie blue G-250 (Gradipore).

Determination of the M_r of the smallest protein was carried out with a 16% gel and standards of 3.4K, 12.5K and 21.5K (Boehringer Mannheim). Glycosylation of the viral proteins was determined by a general glycan staining procedure with reagents supplied by Boehringer Mannheim. N termini of proteins were sequenced in an Applied Biosystems 477A gas phase sequencer after SDS-PAGE and blotting onto membranes using procedures described by Matsudaira (1989).

Nucleic acid characterization. RNA was extracted from capsids with phenol-chloroform (50:50) and was then precipitated with ethanol. To determine its nature, HaSV nucleic acid was digested with RNase A or DNase I (Boehringer Mannheim) under conditions described by the supplier and using ssDNA, dsDNA and ssRNA as controls. Denaturing agarose gel electrophoresis in the presence of formaldehyde was performed according to Sambrook *et al.* (1989).

Polyadenylation status of the viral RNA was determined by two methods. The first compared binding of identical amounts (20 μ g) of viral RNA and poly(A)-selected RNA from *Heliothis virescens* to a 1 ml slurry containing 5 mg of oligo(dT)-cellulose (Pharmacia) in a binding buffer of 20 mM-Tris-HCl pH 7.8, 500 mM-NaCl, 1 mM-EDTA and 0.04% SDS (Sambrook *et al.*, 1989). The second method was by observation of specific priming of viral RNA, and viral RNA polyadenylated with poly(A) polymerase (Pharmacia), with d(T)₁₆A/C/G primers in RNA sequencing reactions using avian myeloblastosis virus reverse transcriptase (U.S. Biochemical) and a protocol provided by the supplier. The 5' cap structure of the genomic RNA of HaSV was determined by observing the ability of polynucleotide kinase to phosphorylate viral RNA before and after preincubation with tobacco acid pyrophosphatase and alkaline phosphatase (Promega) under conditions described by the suppliers. The molar ratio of HaSV strands was determined by quantitative densitometry using a Hoefer GS 300 densitometer on negatives of fluorograms of varying amounts of HaSV RNA resolved on denaturing gels. Cross-linking of RNAs to assess possible co-encapsidation was attempted using the methodology of Newman & Brown (1978).

In vitro translation of HaSV RNA. *In vitro* translation of HaSV RNA was performed with lysates of both rabbit reticulocytes and wheatgerm (Promega) as directed by the supplier. The two viral RNAs were extracted from gel slices after resolution on non-denaturing low melting point agarose gels in Tris-acetate-EDTA (Sambrook *et al.*, 1989).

Results and Discussion

Most well characterized, non-enveloped insect viruses with single-stranded RNA genomes belong to three recognized taxonomic groups, the *Picornaviridae*, the *Nodaviridae* and the *Tetraviridae* (Adams, 1991; Francki & Fauquet, 1991). The remaining viruses are yet to be classified owing to the lack of requisite data or apparent unique features (Adams & Guzo, 1991). Knowledge of the characteristics and biology of picornaviruses and nodaviruses is relatively advanced because of the extensive study of representative viruses that are able to replicate in cell culture. Lack of cell culture systems in which to propagate the other non-enveloped small RNA viruses has restricted their study to examination by electron microscopy, characterization of pathogenicities and physicochemical properties, and analyses of *in vitro* translations of their genomes. Nevertheless, data resulting from these types of studies, such as those presented in this paper, are usually sufficient to allow their comparison with the known virus groupings and thus make an initial classification (Longworth, 1983).

Particle characterization and serology

The buoyant density of HaSV particles in CsCl at pH 7.4 was 1.296 g/ml. They appeared isometric and 38 nm in diameter when negatively stained with uranyl acetate (Fig. 1). The A_{260}/A_{280} ratio of HaSV particles was 1.22, indicating a nucleic acid content of approximately 7% (Gibbs & Harrison, 1976). Reciprocal double-immunodiffusion experiments with HaSV and the N ω V showed

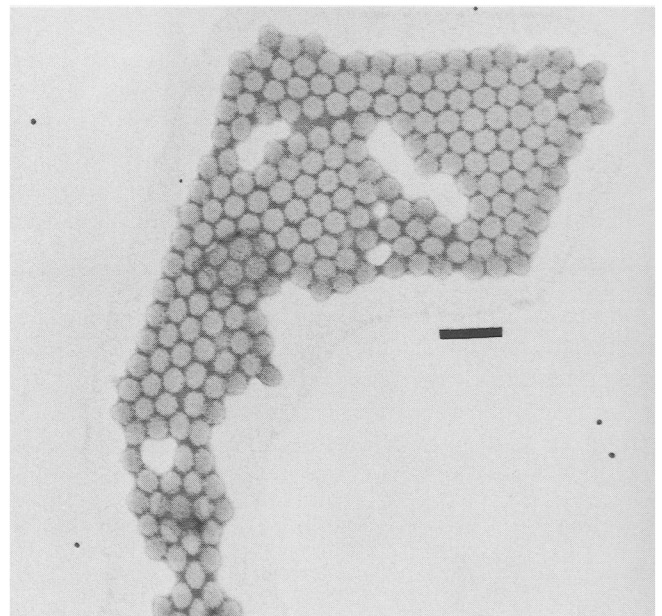


Fig. 1. Electron micrograph of negatively stained HaSV particles. Bar marker represents 100 nm.

no serological relationship. The more sensitive technique of immunoblotting confirmed a lack of antigenic relationship (data not shown). In addition, HaSV did not react with antisera to the N β V in an immunodiffusion test or when immunoblotted. However, no N β V was available as a positive control in these latter experiments. The HaSV particles fluoresced red–orange when stained with acridine orange and irradiated with 310 nm u.v. light, indicating a single-stranded genome.

Protein characterization

Examination of HaSV capsid proteins by SDS–PAGE showed variable results depending on the quantity of protein present. At low protein loadings, two major proteins with M_r s of 65000 and 6000 were evident (Fig. 2, lane 1). However, when more protein was present on the gels, at least 12 further bands with M_r s ranging between 15000 and 70000 became evident (Fig. 2, lane 2). Data from an immunoblotting experiment (Fig. 2, lanes 3 and 4) suggest that the majority of these minor constituents are likely to be degraded forms of the 65K protein. All but the major 6K protein and a minor 15K protein reacted with antibodies specific for the 65K capsid protein. In addition, the minor 70K protein also reacted with the antiserum. The capsid proteins were shown to be non-glycosylated because they failed to react with a hydrazine analogue after oxidation with periodic acid (data not shown).

The N terminus of the 65K protein was blocked, as two efforts to conduct an Edman degradation on the

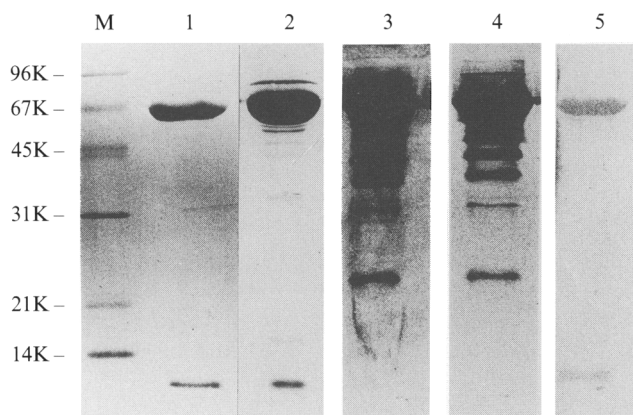


Fig. 2. Analysis of HaSV capsid proteins. Purified particles of HaSV were subjected to SDS–PAGE under reducing conditions in a 12% polyacrylamide gel and then stained with Coomassie blue. Lane M, M_r standards of indicated sizes; lane 2, 0.0003 A_{260} units of HaSV; lane 3, 0.001 A_{260} units of HaSV. Immunoblots are identical when probed with untreated polyclonal rabbit antisera (lane 3) and antibodies mono-specific for the large capsid protein (lane 4); lane 5 is a blot of the capsid proteins treated with a general protein stain and demonstrates the presence of the non-immunogenic small capsid protein on the blot.

resolved and blotted protein failed. After the second attempt, the sample was treated with *N*-chlorosuccinimide and the protein was shown to be present in a quantity normally adequate for sequencing (Felix & Terkelsen, 1973). Fragmentation of the 65K protein was then performed with cyanogen bromide and a 20K fragment was resolved, blotted and sequenced. The 20-residue N-terminal sequence of this fragment was PTLVDNGFWIGGNYALTPTS. The N terminus of the 6K protein was not blocked and an unambiguous 16-residue sequence of FAAAVSAFAANMLSSV was readily obtained.

Nucleic acid characterization

Nucleic acid extracted from HaSV particles was readily hydrolysed by RNase A, but not by DNase I. Denaturing agarose gel electrophoresis of the extracted HaSV RNA genome showed two strands with lengths of 5.5 kb and 2.4 kb (Fig. 3). The RNA strands were shown to lack extensive regions of polyadenylation; only 24% of viral RNA bound to the oligo(dT)–cellulose matrix as compared to 82% of poly(A)–selected RNA. Further evidence for non-polyadenylation of the two viral strands was provided by the observation that the oligonucleotide primer, d(T)₁₆G, gave a clear sequencing ladder when using reverse transcriptase only after *in vitro* polyadenylation of the strands with poly(A) polymerase (data not shown).

The demonstration that the strands could be modified with poly(A) polymerase also showed the lack of any pre-existing 3' modification. The 5' termini of the viral strands were shown to be capped, probably with m⁷G(5')ppp(5')G, as they could not be labelled with polynucleotide kinase and [γ -³²P]ATP unless pretreated with tobacco acid pyrophosphatase and alkaline phosphatase (data not shown). These results show that the genome of HaSV differs in two major aspects from those of the nodaviruses, the only other described group of bipartite small RNA viruses that infect animals. The sizes of the two ssRNA strands (2.4 kb and 5.5 kb) of HaSV are much larger than the 1.4 kb and 3.2 kb strands of Nodamura virus, the nodavirus with the largest genome (Hendry, 1991). Moreover, the strands of HaSV lack the blockage of unknown structure at the 3' termini, as found in the *Nodaviridae*. The characteristics of non-polyadenylation and a 5' capped structure, however, are common to both HaSV and the nodaviruses.

In vitro translation

In vitro translation of viral RNA yielded different results in the two translation systems (Fig. 4). The 5.5 kb RNA, termed RNA1, translated well in the wheatgerm system

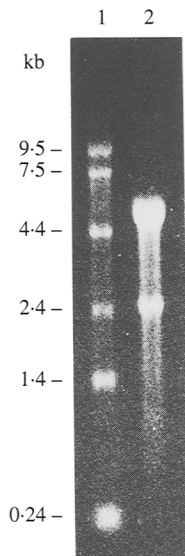


Fig. 3. Analysis of HaSV RNA. The ssRNA genome of HaSV was extracted from particles, electrophoresed on a 1% agarose/formaldehyde gel and visualized with ethidium bromide. Lane 1, RNA standards of indicated length; lane 2, HaSV RNA.

producing more than 20 proteins ranging in size from M_r 195000 to M_r 12000. However, when RNA1 was translated in the reticulocyte system, a large amount of high M_r label was seen in a smear with only a faint band at M_r 200000. The resolution of this translation did not improve with the use of lower percentage gels or other attempts with reticulocyte translations (data not shown). The 2.4 kb viral RNA strand, termed RNA2, yielded a major protein with an M_r 24000 in both systems and a minor protein of M_r 70000. A time course study of translation of the 5.5 kb RNA1 showed that all labelled proteins were produced at similar rates, indicating that the smaller products did not arise through processing of the larger ones (data not shown). When a time course translation experiment (0 to 60 min) was done with the smaller 2.4 kb RNA2, the 24K protein appeared before the 70K protein. No evidence was seen of processing of the 70K protein.

The translation experiments do not give a clear indication of the replication strategy of HaSV. The wheatgerm system demonstrated synthesis of proteins of relative molecular masses near the coding capacity of both RNA strands (200K and 90K for the larger and smaller strands, respectively). In addition, there are a number of proteins encoded by RNA1 with smaller M_r s, the sum of which exceeded the coding capacity of the strand. This observation is consistent with HaSV, during its replication, producing precursor polyproteins which are then cleaved into mature viral proteins. However, no evidence for processing of the *in vitro* translated 195K and 70K proteins of HaSV was seen which would suggest

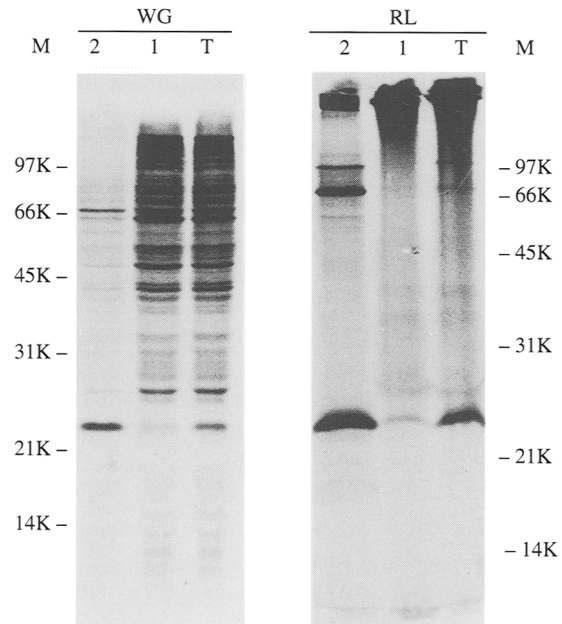


Fig. 4. *In vitro* translation of HaSV genome. The translation products obtained from 0.5 μ g of the 5.5 kbp HaSV strand (RNA1, lane 1), 0.5 μ g of the 2.4 kbp strand (RNA2, lane 2), or 1 μ g of total virus RNA (lane T) were analysed by SDS-PAGE on 14% gels after translation in a wheatgerm extract (WG) or lysate of rabbit reticulocytes (RL) as described. When resolved on a 10% gel, a faint band was evident in the dark smear at M_r 200000 in the translation of RNA1 with RL (data not shown). M, M_r standards.

a replication strategy similar to that of the picornaviruses or comoviruses; the smaller proteins could arise from early termination of synthesis. Should the mature structural proteins of HaSV be processed from larger proteins, a possible candidate for the precursor is the 70K protein translated from the 2.4 kb RNA strand in both systems.

Presence of another form of HaSV

During purification of HaSV particles on CsCl gradients, a minor band with a buoyant density of 1.333 g/ml appeared frequently but in minor, varying amounts (always < 5% of the major band). On four occasions particles from this minor band were fed to *H. armigera* larvae which subsequently showed the same symptoms as those fed with the less dense particles; when the larvae were processed as described above, the 1.296 g/ml HaSV band was again recovered in great excess to a varying but minor amount of the more dense band. In these tests, no virions of either type were recovered from uninfected control larvae. Proteins extracted from the more dense particles appeared identical to those from the less dense particles when examined by SDS-PAGE and immunoblotting with antibodies specific for the 65K protein of HaSV (data not shown). Furthermore, the more dense

particles contained two RNA strands of the same size as the 5.5 and 2.4 kb strands contained in the less dense HaSV particles. When the particles from the more dense band were examined by electron microscopy, they appeared to have a diameter of 45 nm but were otherwise highly similar to the 38 nm particles (data not shown). Two different sized particles with the same diameters were also seen in electron micrographs of diseased tissue (K. C. Binnington, P. Christian & E. Brooks, unpublished).

Establishing the exact origin of the more dense form of HaSV detected in CsCl gradients will require further work. Possibilities include an instability to chloride ions similar to that observed in nodaviruses (Hendry, 1991) or alternative packing of the RNA genome into HaSV capsids. The latter possibility raises the intriguing question of whether both strands of the HaSV genome are encased in the same capsid. An interesting dichotomy between unenveloped plant and animal viruses with multipartite single-stranded RNA genomes is the observation that plant viruses encase their strands in different capsids, whereas the sole group of animal viruses of this type (excluding HaSV and like viruses), the nodaviruses, encase theirs in the same capsid (Kaesberg, 1987). Two lines of evidence suggest the RNA strands of the HaSV genome are not encased in a manner similar to the nodaviruses. Firstly, the molar ratio of the RNA strands is not unity, as determined by quantitative densitometry of fluorograms of the resolved strands. Densitometry of six gel lanes with different loadings showed the ratio of the amount of RNA in the smaller strand to the amount in the larger strand to be 1:1.7 (s.d. ± 0.24). This ratio is significantly different to that of the equimolar ratio of 1:2.25, and suggests that HaSV particles encapsidate a molar excess of the smaller RNA strand. Secondly, attempts to cross-link the strands by u.v. irradiation, using a procedure (Newman & Brown, 1978) similar to that used for a nodavirus, failed. Clearly, further work is needed to resolve the question of how the strands of the HaSV genome are encapsidated.

The characteristics we report for HaSV match most closely with those of the *Tetraviridae*, formerly known as the *Nudaurelia* β group of viruses and include buoyant densities, capsid sizes, non-polyadenylation of the genome, and M_r s of the capsid protein components. Members of this group of seven recognized and 10 provisionally classified viruses have been isolated exclusively from lepidopteran insects (Reinganum, 1991; Moore, 1991*a*). The family name is derived from the group's characteristic icosahedral capsid which is composed of multiple copies of a single protein subunit arranged with $T = 4$ symmetry. The type member, N β V, has a monopartite genome of approximately 5 kb encased in a 38 nm capsid (density in CsCl is 1.3 g/ml;

Moore, 1991*a*) which was originally reported to be composed of a single major protein constituent of 60K. However, recent work has shown the presence in the capsid of a small protein of M_r approximately 8K (Agrawal & Johnson, 1992).

An anomalous provisional member of the *Tetraviridae* is N ω V. This virus shows a particularly close relationship to HaSV and was reported recently as having a 40 nm capsid composed of two 62K and 8K protein constituents and also a bipartite genome with ssRNA strands of approximately 5 kb and 2.5 kb (Agrawal & Johnson, 1992; Moore, 1991*a*). The capsid structure of N ω V was also shown to have $T = 4$ symmetry (Cavarelli *et al.*, 1991). Sequencing the smaller 2.5 kb RNA of N ω V demonstrated the potential to encode the coat proteins' precursor that is cleaved at a Asn-Phe site 74 residues from its C terminus (Agrawal & Johnson, 1992). Predicted amino acid sequences at the N terminus of the 8K protein (FAAAVLAFANMLTSV) and an internal fragment of the 63K protein (PTLVDNGFWIGGNYALTPTS) are 88% and 94% identical, respectively, to the HaSV sequences we report. Interestingly, the strong sequence relationship between the capsids of N ω V and HaSV is not reflected serologically.

From the available data, it would appear that HaSV and N ω V form a previously unrecognized group with the provisional suggestion that a new genus be formed within the *Tetraviridae*. Indeed, precedents exist for the formation of two genera within a family that possess different numbers of genome segments (i.e. the cypovirus and coltivirus genera in the *Reoviridae*). However, we believe that insufficient data are available on the replication strategy of the *Tetraviridae* and its typemember, N β V, as well as HaSV (or N ω V) to warrant this action. Should the two groups have distinct means of replicating themselves the formation of a different family would be justified, despite both having the unusual $T = 4$ symmetry in their capsids. This course of action would be similar to that with viruses having capsids with the more common $T = 3$ symmetry. We therefore caution against the formation of a new group until further data become available.

The pathogenicity of HaSV to an economically important insect pest and the apparent specificity of its group to lepidopteran insects (Reinganum, 1991) raise the possibility that HaSV may be of use in agriculture. Small RNA viruses pathogenic to insects are not generally thought of as viable biological control agents for agricultural insect pests as their use in the field is hindered by reasons that include beliefs about their instability and concern over their relatedness to vertebrate viruses. There are, however, at least three instances in the literature where small RNA viruses have

been effective against pest insects. The *Nudaurelia* complex of small RNA viruses has caused large epizootics in populations of the pine tree emperor moth (*N. cytherea capensis* Stoll). The *Darna trima* virus, also a tetravirus, was used successfully against a moth pest of oil palms in Malaysia (Moore, 1991 *a*), and a picorna-like virus, the *Gonometa* virus, controlled a moth pest of pines in Uganda (Moore, 1991 *b*). It may be that a closer look at HaSV and other entomopathogenic small RNA viruses, and the development of new strategies for their use, will make these viruses more useful to agriculture (Christian *et al.*, 1992).

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