Providence virus: a new member of the Tetraviridae that infects cultured insect cells

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Abstract

We identified a new member of the Tetraviridae, Providence virus (PrV), persistently infecting a midgut cell line derived from the corn earworm (Helicoverpa zea). Virus purified from these cells also productively infected a H. zea fat body cell line, and a cell line from whole embryos of the beet armyworm, Spodoptera exigua. PrV is thus the first tetravirus shown to replicate in cell culture. PrV virions are isometric particles composed of two structural proteins (60 and 7.4 kDa) that encapsidate both the genomic (6.4 kb) and the subgenomic (2.5 kb) RNAs. The monopartite organization of the PrV genome resembles that of Nudaurelia /H9252 virus and Thosea asigna virus, members of the genus Betatetravirus. The predicted sequence of the PrV structural proteins demonstrates homology to tetraviruses in both genera. The infectivity of PrV for cultured cells uniquely permitted examination of tetravirus RNA and protein synthesis during synchronous infection. The discovery of PrV greatly facilitates studies of tetravirus molecular biology.

Keywords: Tetravirus; Tetraviridae; Cell culture; Insect virus; Providence virus; RNA virus; T = 4

Introduction

Tetraviruses originally attracted interest about 40 years ago because of their ability to cause epizootics in pest insect species (Grace and Mercer, 1965; Hendry et al., 1968). Viruses in this family exclusively infect lepidopteran insects (butterflies and moths) and are extremely host-specific, usually confined to a few closely related species (Hanzlik and Gordon, 1997). Tetraviruses have single-stranded positive-sense RNA genomes. The complete nucleotide sequences have been determined for two members of the family, including species from the two genera: Nudaurelia /H9252 virus (N/H9252V; Betatetravirus) (Gordon et al., 1999) and Helicoverpa armigera stunt virus (HaSV; Omegatetravirus) (Gordon et al., 1995; Hanzlik et al., 1995). The genome organization differs between these genera (Hanzlik and Gordon, 1999). Betatetraviruses such as NβV and Thosea asigna virus (TaV) have monopartite genomes that contain about 6.5 kb. The RNA-dependent RNA polymerase (RdRp) is expressed from the genomic RNA, and the structural protein is expressed from a 2.5-kb subgenomic transcript which is encapsidated in virions (Gordon et al., 1999; Pringle et al., 1999). In contrast, the omegatetraviruses Nudaurelia o virus (NoV) and HaSV have bipartite genomes composed of two unique RNA molecules: a 5.3-kb RNA that encodes the RdRp and a separate 2.5-kb RNA that encodes the structural proteins (Agrawal and Johnson, 1992; Hanzlik et al., 1993).

Tetraviruses are also interesting from a structural perspective. The name tetravirus derives from the T = 4 capsid architecture of members of this family, currently the only nonenveloped viruses known to have this symmetry (Hanzlik and Gordon, 1999). The structure of NoV has been solved by both X-ray crystallography and electron cryomicroscopy (cryo-EM) (Munshi et al., 1996, 1998), and
that of NBV has been examined by transmission electron microscopy (TEM) and cryo-EM (Finch et al., 1974; Olson et al., 1990). Each tetravirus particle is composed of 240 copies of two structural proteins that range in size from 56 to 63 kDa and from 6 to 8 kDa, respectively (Ball et al., 2000). The structural proteins are produced by proteolysis of a capsid precursor in an autocatalytic postassembly cleavage reaction (Agrawal and Johnson, 1992, 1995). In the case of TaV, processing of the capsid precursor is predicted to yield a 17-kDa nonstructural protein of unknown function in addition to the large and small capsid proteins (Pringle et al., 1999, 2001). Recent studies of NoaV virus-like particles made using a baculovirus expression system have shown that during maturation NoaV provirions undergo large conformational changes (Canady et al., 2000, 2001).

Despite the advent of molecular techniques that have begun to allow the characterization of tetraviruses, their inability to replicate in cell culture severely hinders these studies. Previous attempts to identify cell lines that support the replication of these viruses were unsuccessful (Hanzlik and Gordon, 1997; Moore and Tinsley, 1982). An extensive study that tested 14 cell lines for susceptibility to HaSV by either infection or transfection of viral RNAs failed to identify a cell line that supported HaSV replication (Bawden et al., 1999). Since there is no cell-culture system, tetraviruses must be either purified from infected larvae or produced by another method. The insect hosts are difficult or impossible to rear in the laboratory (Hanzlik and Gordon, 1997), and heterologous systems for production have thus far been unable to produce significant quantities of virus (Gordon et al., 2001; Venter, 2001). Therefore, several aspects of tetravirus replication remain poorly characterized.

This article describes the isolation of a new virus that we discovered persistently infecting a midgut cell line (MG8) derived from the corn earworm, Helicoverpa zea. Virus was purified from these cells and used to infect two other cell lines, one from H. zea fat body (Kariuki et al., 2000) and one from larvae of the beet armyworm, Spodoptera exigua (Gelernter and Federici, 1986). We present evidence that this virus is a previously undescribed tetravirus. Because of the uncertainty about its natural host range, we have named this new tetravirus Providence virus (PrV) derived from the location of the laboratory where the MG8 cell line was generated. Since it replicates in cell culture, PrV provides a unique opportunity to examine aspects of tetravirus biology that have previously been out of reach. We have taken a first step toward elucidating the life cycle of a tetravirus by studying PrV macromolecular syntheses during synchronous infection of FB33 cells.

Results and discussion

Isolation of a virus persistently infecting a H. zea midgut cell line

RNA molecules that are synthesized in a DNA-independent manner, such as the products of viral RdRps, can be specifically radiolabeled by incorporation of [3H]uridine in the presence of actinomycin D. In the absence of deliberate infection with an RNA virus, most cell lines show only minimal background labeling (Fig. 1, Lane 1; uninfected BCIRL-HZ-FB33 cells; referred to here as FB33). Infection with an RNA virus, such as the nodavirus Pariacoto virus (PaV), reveals the production of virus-specific bands by RNA-dependent RNA synthesis (Fig. 1, Lane 2; PaV-infected FB33 cells). However, in the BCIRL-HZ-MG8 cell line (referred to here as MG8), two prominent RNAs were detected even in uninfected cells (Fig. 1, Lane 3). These cells were originally derived from the midgut of larvae from a laboratory colony of the corn earworm, H. zea (as described under Materials and methods). MG8 cells were susceptible to infection by PaV (Johnson and Ball, 2001), which provided 3.0- and 1.3-kb RNAs to use as size markers (Fig. 1, Lane 4). By comparison with these RNAs and the 6.0-kb dimer of PaV RNA1 (Johnson et al., 2000), the unidentified RNAs present in untreated and PaV-infected MG8 cells were estimated to be 6.4 and 2.5 kb. The size of the larger RNA was also estimated to be 6.4 kb by com-

Fig. 1. Products of RNA replication detected in cell lines derived from Helicoverpa zea. FB33 cells (Lanes 1 and 2) and MG8 cells (Lanes 3 and 4) were infected with PaV (Lanes 2 and 4) or left untreated (Lanes 1 and 3); 24 h.p.i. (Lanes 1 and 2) and 72 h.p.i. (Lanes 3 and 4) RNAs were metabolically labeled by incorporation of [3H]uridine in the presence of actinomycin D. Total cellular RNAs were extracted, resolved by electrophoresis on denaturing 1% agarose-formaldehyde gels, and visualized by fluorography. The size of the two PaV genomic RNAs (3.0 and 1.3 kb) are indicated on the right, as are the estimated sizes of the RNAs identified in MG8 cells.
parison to an RNA molecular weight marker on ethidium bromide stained gels (data not shown). In addition to the unexpected RNAs seen in MG8 cells, when concentrated spent medium from very early passage MG8 cells (passage 8) was analyzed by SDS–PAGE and isoelectric focusing, a major protein of approximately 60 kDa with an acidic pI (4.7) was the major product detected (Goodman and McIntosh, 1995). Although no previously described tetravirus has been reported to replicate in cultured cells (Bawden et al., 1999; Moore, 1991), these RNA and protein sizes are characteristic of tetraviruses (Gordon and Hanzlik, 1998). Furthermore, all known tetraviruses exhibit tropism for the midgut of lepidopteran insects (Hanzlik and Gordon, 1997), the source of the MG8 cell line. These circumstances suggested that MG8 cells might carry a persistent tetravirus infection and prompted us to attempt virus isolation.

To determine whether infectious virus could be recovered from MG8 cells, a virus purification procedure that had been successfully used for the isolation of tetraviruses from insects (Pringle et al., 1999) was adapted for virus purification from cultured cells. Briefly, MG8 cells were lysed with a nonionic detergent and the lysate was pelleted through a 30% sucrose cushion. Virus was purified by centrifugation on 10–40% sucrose gradients, and gradient fractions were analyzed for the presence of putative viral proteins by SDS–PAGE. A protein of approximately 60 kDa was consistently detected by Coomassie blue staining (Fig. 2A). Fractions containing the 60-kDa protein were combined and after further purification, the resulting preparation was characterized. Matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry analysis of purified virus identified a small protein with a mass of 7439 Da (Fig. 2B), which was also detected when virions were examined by tricine SDS–PAGE (data not shown). The sizes of the two structural proteins closely resemble the capsid proteins of other tetraviruses, which range in size from 56 to 63 and 6 to 8 kDa for the large and small capsid proteins, respectively (Gordon and Hanzlik, 1998).

When sucrose gradient-purified material was negatively stained and examined by TEM, nonenveloped isometric particles with a diameter of about 40 nm were visualized (Fig. 2C). Their structure was examined at higher resolution by cryo-EM and 3D image reconstruction (P. Natarajan and J.E. Johnson, Scripps Research Institute, personal communication). The virions have an external capsid architecture that resembles that of NbV and TaV (Olson et al., 1990; Pringle et al., 1999). Twelve capsomeres are arranged as four Y-shaped trimers on each triangular face of the virion, yielding a $T = 4$ icosahedral lattice composed of 240 subunits per virion. Each face contains three distinct pits, and adjacent faces are separated by deep surface grooves. A detailed description of the structure will be presented elsewhere.

The large and small capsid proteins from purified virions were resolved on tricine SDS–PAGE gels, transferred to PVDF membrane, and excised separately. Their N-terminal sequences were determined to be PQMT... and FAGTV..., respectively. Both amino acid sequences were identified in a single open reading frame (ORF) of 2265 nt in a partial cDNA clone of PrV RNA. The sizes predicted

![Fig. 2. Characterization of virus purified from MG8 cells. Virus was purified from MG8 cells on 10-40% sucrose velocity gradients and fractions collected. (A) The proteins present in each fraction were resolved by SDS–PAGE on 10% gels and visualized by staining with Coomassie blue. Fractions 1 to 5 (numbered from the bottom of the gradient) of a total of 10 are shown. The sizes of the molecular mass markers are shown on the left. The star in Lane 4 indicates the putative capsid precursor protein. The fractions containing the 60-kDa protein were combined and analyzed further. (B) MALDI-TOF mass spectrometry analysis was performed directly on the purified sample. The spectrum is shown for the region in which a protein of 7439 Da was identified. (C) The purified sample was negatively stained and analyzed by TEM. The diameter of the virus particles was estimated to be 40 nm. The size bar represents 100 nm.](image-url)
for the large and small capsid proteins from the nucleotide sequence of the cDNA were 60,649 and 7434 Da, respectively, which correlate well with the results of SDS–PAGE and MALDI-TOF analysis of the virion proteins. Moreover, the pl of the 60.6-kDa capsid protein predicted from its ORF (4.78) agreed well with the experimentally determined value of 4.7 (Goodman and McIntosh, 1995).

Within the ORF, the N-terminus of the large capsid protein (PQMT...R ...) was preceded by 123 codons that are predicted to encode a protein of 13.4 kDa. Other tetraviruses have two different strategies of capsid protein expression. In NβV, NaoV, and HaSV, the large and small capsid proteins are generated by cleavage of the capsid protein precursor at a single site (Agrawal and Johnson, 1992; Gordon et al., 1999; Hanzlik et al., 1993), whereas in TaV, the precursor is cleaved twice to produce a nonidentical nonstructural protein in addition to the large and small capsid proteins (Pringle et al., 1999). The presence of 123 codons preceding the start of the PrV large capsid protein suggests that it may have a processing strategy similar to that of TaV.

The predicted amino acid sequence of the structural portion of the PrV capsid protein precursor (from the proline residue at the N-terminus of the large capsid protein to the C-terminus of the small capsid protein) was compared to the structural protein sequences of other tetraviruses. The results, presented as a similarity/identity matrix of pairwise alignments in Fig. 3, clearly established that PrV is related to the four other tetraviruses that have been characterized in this detail. The structural proteins of PrV were somewhat more similar to those of the omegatetraviruses, HaSV and NaoV (47 and 46%, respectively), than to those of the betatetraviruses, TaV and NβV (35% to both). The sizes of the viral RNAs and proteins, the T = 4 icosahedral symmetry of the nonenveloped virus particles, and the definitive homology of the structural protein sequences with those of other tetraviruses, all support the inclusion of this virus in the family Tetraviridae.

The genome organization of PrV resembles that of NβV and TaV

During the course of this study, MG8 cells from both a low passage (passage 32) and a high passage (passage 100+) of the cell line were examined for the presence of PrV. Virus was purified from both low- and high-passage cells, indicating that PrV persistently infected MG8 cells even after numerous passages. Preparations of virus from both low- and high-passage MG8 cells were found to be infectious (see below and data not shown). The presence of a major protein species of 60 kDa in concentrated spent medium from very early passage MG8 cells (passage 8) (Goodman and McIntosh, 1995) further suggests that PrV was present in these cells soon after the cell line was established and may have been present in the laboratory colony of H. zea from which the cell line was prepared. The tetravirus HaSV was also discovered as an infection in a laboratory colony of H. armigera (Hanzlik et al., 1993).

Viruses in the Betatetravirus genus (such as NβV and TaV) have monopartite genomes and express their structural proteins via a subgenomic RNA. Those in the Omegatetravirus genus (such as NaoV and HaSV) have bipartite genomes in which the larger genome segment (RNA 1) encodes the RdRp and the smaller segment (RNA 2) encodes the capsid protein precursor (Gordon and Hanzlik, 1998). Although NβV and TaV have monopartite genomes, both the genomic and the subgenomic RNAs are encapsidated (Gordon et al., 1999; Pringle et al., 1999).

To examine the genome organization of PrV, total RNA from MG8 cells and RNA isolated from purified virions were analyzed by Northern blot hybridization. Two cDNA clones that contained small portions (544 and 705 nt) of the PrV genome were generated during this study. The 544-nt cDNA contained the 3’ end of the ORF that encodes the capsid proteins, whereas the 705-nt cDNA represented a portion of the nonstructural region of the PrV genome (data not shown). We anticipated that if PrV had a bipartite genome similar to omegatetraviruses, probes made from the 705- and 544-nt cDNAs would hybridize uniquely to the large and small RNA molecules, respectively. However, if PrV had a monopartite genome that transcribed a subgenomic RNA, the 544-nt probe would hybridize to both RNA species. Figure 4A shows that the probe from the 544-nt cDNA hybridized to both the 6.4- and the 2.5-kb RNAs. The probe from the 744-nt cDNA hybridized only to the larger RNA molecule (Fig. 4B). Both RNAs were encapsidated in virus particles (Fig. 4, virion lanes). These results established that PrV has a monopartite genome organization similar to NβV and TaV. As is the case for NβV and TaV, the 2.5-kb subgenomic RNA of PrV is encapsidated in virions.
particles per cell at the highest dose of virus to approx-
of infection (m.o.i.s) that ranged from approximately 1
bolically labeled with \([3\text{H}]\)uridine in the presence of acti-
pathic effect was evident by phase contrast microscopy at
approximately 10 particles per cell at the lowest dose. No cyto-
virus genera are shown for comparison.

Fig. 4. Analysis of the genome organization of PrV. RNA was extracted
from MG8 cells (Lanes 1 and 3) or purified virus (Lanes 2 and 4), resolved
on denaturing 1% agarose-formaldehyde gels, and transferred to nylon
membranes. The membranes were hybridized with riboprobes transcribed
from cDNA clones containing small portions (544 nt, A; 705 nt, B) of the
PrV genome. The probe generated from the 544-nt clone hybridized only to the genomic (6.4 kb) and the subgenomic (2.5 kb) RNAs, whereas the probe from the 705-nt clone hybridized only to the genomic RNA. The
sizes of the RNAs are indicated on the left. Schematic diagrams of the
genome organization of viruses from the Betatetravirus and Omegatetrav-
irus genera are shown for comparison.

Infectivity of PrV for other cell lines

We tested the infectivity of PrV in a cell line derived from the fat body of \(H.\) \(zea\) larvae (FB33) (Kariuki et al.,
2000) and in a cell line derived from \(S.\) \(exigua\) larvae (Se-1)
(Gelernter and Federici, 1986). Cells in six-well plates were infected with serial 10-fold dilutions of purified PrV from 3
\(\mu\)g per well to 3 \(\times 10^{-4}\) \(\mu\)g per well, yielding multiplicities of
infection (m.o.i.s) that ranged from approximately 1 \(\times\)
\(10^{3}\) particles per cell at the highest dose of virus to approxi-
mately 10 particles per cell at the lowest dose. No cyto-
pathic effect was evident by phase contrast microscopy at
any dose in either cell line. At 48 h p.i., cells were meta-
bolically labeled with \([3\text{H}]\)uridine in the presence of acti-
nomycin D; then total cellular RNA was extracted, resolved
by electrophoresis on denaturing agarose-formaldehyde
gels, and visualized by fluorography. RNA molecules cor-
responding in size to the 6.4-kb genomic and 2.5-kb sub-
genomic PrV RNAs were labeled in both cell types. Since
input RNA is not detected using this technique, this result
demonstrates that both FB33 and Se-1 cells were suscepti-
bles to PrV infection (data not shown). PrV RNAs were
detected in FB33 cells at m.o.i.s as low as 1 \(\times 10^{3}\) particles
per cell, but in Se-1 cells only at the highest m.o.i. We
therefore chose FB33 cells as the host cell system for further
analysis of PrV infection.

Synthesis of viral RNAs and proteins following
synchronous infection

Cell culture provides ready access to virus replication
cycles. Synchronous infections of FB33 cells were used as a first step toward analysis of RNA and protein production
during a PrV infection. The time course of infection was
analyzed by the following: (1) Northern blot hybridization
to assess the accumulation of positive- and negative-sense
genomic and subgenomic RNA molecules (Figs. 5A and
5B); (2) metabolic labeling of RNA to analyze RNA syn-
thesis (Fig. 5C); and (3) Western blot to assay the accumu-
lation of structural proteins (Fig. 6).

Input positive-sense genomic and subgenomic RNA
were detected by Northern blot hybridization at 6 and 12 h
p.i. (Fig. 5A). There was a significant increase in the amount
of RNA detected between 18 and 24 h p.i., and it reached a
maximum at about 36 h p.i. (Fig. 5A). By metabolic label-
ning, RNA synthesis was first detected at 18 h p.i. (Fig. 5C).
The synthesis of the subgenomic RNA slightly lagged that
of the genomic RNA. Negative-sense RNAs were also not
detected until 18 h p.i., when both the genomic and the
subgenomic RNAs were observed (Fig. 5B). At 36 h p.i.
these negative-sense RNAs reached peak levels that were
about 10-fold lower than the corresponding positive-sense
species (Fig. 5B). This is the first time that negative strands
have been detected for a tetravirus subgenomic RNA, and it
raises the possibility that the PrV subgenomic RNA can be
replicated. Evidence for replication of the subgenomic RNA
of the nodavirus \(Flock\) \(house\) \(virus\) has recently been pre-
sented (L. D. Eckerle and L. A. Ball, unpublished results),
but this issue needs further study. Positive- and negative-
sense RNA accumulation monitored over 5 days of infec-
tion mirrored the results seen in the shorter time course, and
RNA accumulation at later time points was maintained at a
similar level to the 48 h samples in the shorter time course
(data not shown).

Total protein from mock-infected and PrV-infected
FB33 cells was analyzed by immunoblotting for the pres-
ence of PrV-specific proteins using a polyclonal antibody
raised in rabbits against purified PrV (Fig. 6). The large
capsid protein of the input virus was detected 6 h p.i.
and this band increased in intensity from about 24 h p.i.
Beginning at 36 h p.i., a larger capsid-specific protein accumu-
lated, having a size appropriate for a precursor comprising
the large and small structural proteins (approximately 68
kDa). Synthesis of this 68-kDa precursor protein slightly
lagged the appearance of the subgenomic RNA that is likely
to direct its translation. Although the 68-kDa precursor to
the large and small capsid proteins accumulated in infected cells, this protein was not detected in purified virions (Fig. 6, lanes V and V+C). In other tetraviruses, immature provirions undergo cleavage of the precursor protein into the large and small capsid proteins that comprise mature virions (Agrawal and Johnson, 1995; Johnson and Reddy, 1998). The absence of the 68-kDa precursor protein in purified PrV may be due to maturation of the virus during purification and/or storage. A small amount of the putative 68-kDa precursor to the large and small capsid proteins was detected in virus samples during purification (Fig. 2A, Lane 4). A species corresponding to the primary translation product of the 2265-nt ORF was not detected either in cells during the time course or in purified virions. However, processing of this precursor could be extremely rapid. Immediately upstream of the proline residue at the N-terminus of the PrV large capsid protein, there is a cis-acting processing signal that is active in a diverse range of viruses (e.g., TaV, Cricket paralysis virus, and Foot-and-mouth disease virus; see Donnelly et al., 2001).

Detection of virus particles in infected cells

To determine whether virions could be observed in infected cells, infected FB33 cells and persistently infected MG8 cells were sectioned and examined by TEM. FB33 cells were infected with purified PrV at an m.o.i. of approximately 1 × 10^4 particles per cell and harvested at 3 days p.i. Persistently infected MG8 cells (passage 100+) were harvested from 75-cm² tissue culture flasks, but were otherwise treated identically to FB33 cells. Sectioned cells were examined by TEM, and their appearance compared between the two cell lines and to mock-infected FB33 cells.

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Virus particles approximately 40 nm in diameter were identified in both cell lines (Fig. 7). In each case, virus particles were detected only in the cytoplasm. The particles were associated with an unidentified cytoplasmic structure, which in some sections appeared to be enclosed by a membrane. TEM of tissues isolated from tetravirus-infected whole larvae have also demonstrated the presence of virus particles within the cytoplasm, often within cytoplasmic vesicles (Moore, 1991; Moore and Greenwood, 1984).

In the sections of MG8 cells examined, almost every cell had at least one virus-containing structure (Figs. 7A and 7B, arrows), and most cells had a number of the structures. Occasionally, a clump of virus particles was observed that was associated with the cell membrane (Fig. 7C), but it was unclear whether this represented viral endocytosis or release.

The abundance of virus particles observed in infected FB33 cells varied more than in MG8 cells. Some FB33 cell sections did not appear to have any virus particles, whereas others had virus-containing cytoplasmic structures similar to those seen in MG8 cells. One to two percent of the FB33 cells examined contained numerous virus particles (Figs. 7D and 7E). In these cells, virus particles were visible in large arrays within cytoplasmic structures and also as individual virus particles spread throughout the cytoplasm. In addition, single virus particles could be identified on the outside of the cell membrane (arrows, Fig. 7E). Mock-infected FB33 cells did not contain any particles resembling virions in the cytoplasm nor did they exhibit cytoplasmic structures that contained particles (Fig. 7F).

Infected Se-1 cells were also sectioned and examined by TEM, but these cells contained very few virus particles, correlating well with the reduced amount of viral RNA detected in these cells compared to the MG8 and FB33 cells (data not shown). However, similar to the virus seen in MG8 and FB33 cells, virus in Se-1 cells was associated with a cytoplasmic structure (data not shown).

**TaV, NβV, and HaSV did not infect FB33 cells**

To determine whether FB33 cells could support infection by other tetraviruses, samples of TaV, NβV, and HaSV were assessed for infectivity by metabolic labeling of RNA. The HaSV preparation used in this study was verified as being pathogenic for its insect host (T. N. Hanzlik, CSIRO, personal communication), although the infectivity of the NβV and TaV preparations could not be verified for technical reasons. Using the method that detected the synthesis of PrV RNAs, no TaV-, NβV-, or HaSV-specific bands were identified in total RNA extracted from cells labeled...
Materials and methods

Insect cell cultures

Insect cell lines derived from H. zea (corn earworm, Lepidoptera, Noctuidae) fat body (BCIRL-HZ-FB33, referred to here as FB33) (Kariuki et al., 2000) and Lepidoptera, Noctuidae) fat body (BCIRL-HZ-FB33, referred to here as MG8), which was developed as described below, was maintained at 28°C in serum-free ExCell-401 medium (JRH Biologicals) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. A H. zea midgut cell line (BCIRL-HZ-MG8, referred to here as MG8), which was developed as described below, was maintained at 28°C in serum-free ExCell-401 supplemented with antibiotics.

Establishment of the MG8 cell line from H. zea

Fourth or fifth instar larvae of H. zea were starved for approximately 4 h and surface sterilized by immersing the larvae in 70% alcohol for 10-15 min followed by rinsing in Hanks’ balanced salt solution (HBSS). The larvae were pinned on sterile wax in a petri plate and dissected, and the fat body, silk gland, tracheae, and Malphigian tubules cleared away. A few milliliters of HBSS was added to the plate and the larvae were immersed in the solution. The outer part of the midgut was raised gently with forceps and a small opening cut in it to allow the extrusion of food. The midgut was cut at both ends and the whole peritrophic membrane and any remaining food discarded. The midgut was then placed in a petri dish in HBSS containing 100 units penicillin/ml and 100 μg/ml streptomycin and then rinsed twice in HBSS without antibiotics. The midgut tissue was cut into small pieces with dissecting scissors and 1-1.5 ml trypsin–EDTA (Sigma) solution added and allowed to incubate at room temperature for 3-5 min. Five milliliters of TC-199MK medium (McIntosh et al., 1973) containing 10% heat-inactivated fetal bovine serum (FBS; 56°C/30 min) was added to the minced tissue. The tissue was removed from the dish and centrifuged at 1000 rpm for 10 min in a Beckman Model TJ-6 table-top centrifuge. The supernatant was discarded and the pellet resuspended in 5 ml ExCell-400 (JRH Biosciences) supplemented with 10% inactivated FBS. The resuspended pellet was transferred to a 25-cm² tissue culture flask. Cultures were routinely re-fed at 10 day intervals until the cells began to proliferate.

In this study, MG8 cells from a low passage (passage 32) and a high passage (passage 100+) were routinely used. Both low- and high-passage MG8 cells were grown in serum-free medium as described above, since it was observed that the presence of 10% FBS caused the cells to become stressed, round up, and detach from the flask surface. Unless otherwise noted, virus or cells used in experiments were derived from passage 32.

Analysis of concentrated spent media from the MG8 cell line

Spent media concentrates from passage 8 MG8 cells were generated by dialyzing spent medium against 0.05 M sodium phosphate buffer (pH 7.0) and then concentrating using Aquacide II. Protein (10–22 μg) from these samples was analyzed by denaturing electrophoresis on 8–25% acrylamide gels using the PhastSystem (Amersham Biosciences) as described by Goodman et al. (1997). Isoelectric focusing using the Multiphor System (Amersham Biosciences) was performed as described previously (McIntosh and Ignoffo, 1983).

RNA labeling, extraction, and analysis

The products of RNA replication were labeled by metabolic incorporation of [3H]uridine for 3 h in the presence of 20 μg/ml actinomycin D as previously described (Ball, 1992). Total RNA was extracted using an RNAgent kit (Promega) according to the manufacturer’s directions. RNAs were resolved by electrophoresis on 1% agarose-formaldehyde gels and visualized by fluorography (Laskey and Mills, 1975). In passage 100+ cells infected with the nodavirus Pariaecotto virus, the genomic RNAs of 3011 and 1311 nt, were used as size standards. The 0.24- to 9.5-kb RNA ladder (Invitrogen) was included as a size standard on gels when RNAs were visualized by staining with ethidium bromide.
**Virus purification from MG8 cells**

MG8 cells were grown as monolayers in tissue culture flasks at 28°C for 5 to 7 days. Cells were lysed by rocking for 15 min at room temperature in the presence of 0.5% NP-40. Cellular debris was removed by centrifugation at 10,000 g for 20 min. Virus was pelleted from the supernatant through a 30% sucrose cushion (in 50 mM sodium phosphate buffer, pH 7.2) by centrifugation at 100,000 g for 2.5 h. Virus pellets were resuspended overnight at 4°C in 50 mM phosphate buffer and then subjected to centrifugation through a 10–40% sucrose velocity gradient, and fractions were collected and analyzed as described previously (Pringle et al., 1999). Virus was repelleted from the pooled gradient fractions and resuspended as described above. Purified virus was stored at −20°C.

**Analysis of gradient fractions by SDS–PAGE**

Gradient fractions were analyzed for the presence of viral proteins by SDS–PAGE. Samples were boiled in 2× sample buffer for 5 min before electrophoresis at 170 V through a 10% glycine SDS–polyacrylamide gel (Laemmli, 1970). Proteins were stained with Coomassie blue. A broad range molecular mass marker (New England Biolabs) was included as a size standard.

**Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry**

Samples were analyzed in the linear mode on a Voyager Elite mass spectrometer with delayed extraction technology (PerSeptive Biosystems, Framingham, MA). The acceleration voltage was set at 25 kV, and 100 to 150 laser shots were summed. Sinapinic acid (Aldrich) dissolved in acetonitrile:0.1% trifluoroacetic acid (1:1) was used as the matrix. Virus samples were diluted 1:10 with the matrix, and 1 μl was pipetted onto a smooth plate. Two internal standards, insulin (5734 Da) and ubiquitin (8565 Da), were included to calibrate detection in the size range of the small capsid protein.

**N-terminal sequencing of PrV structural proteins**

Purified virions were denatured and electrophoresed through a 10% tricine SDS–PAGE gel (Schagger and von Jagow, 1987). Proteins were transferred to Immobilon P membrane (Millipore) by semidyration blotting with the Millipore Graphite Electroblotter System. Proteins were visualized on the membrane by staining with 0.025% Coomassie blue in 40% methanol for 2 min. The membrane was destained with 50% methanol and then washed in deionized water. Bands corresponding to the large and small capsid proteins were excised. Five cycles of sequencing were performed on each protein using a Beckman Model PI 2090E sequencer.

**Determining the similarity of the capsid precursor protein of PrV to those of other tetraviruses**

The sequence of a 2593-nt cDNA clone containing a portion of the PrV genome was determined using fluorescent dideoxynucleotides in an automated DNA sequencer and analyzed using GCG software for the presence of ORFs. A 2265-nt ORF was identified that encoded the capsid precursor protein (GenBank Accession No. AF548354). The structural protein sequences predicted from this ORF were compared to the capsid protein sequences of TaV (GenBank Accession No. AF062037), HaSV (NC_001982), NβV (NC_001990), and NαV (S43937). Pairwise alignments were performed in GCG using GAP, with a gap creation penalty of 8 and a gap extension penalty of 2. The similarity and identity scores from these alignments were used to prepare the similarity matrix shown in Fig. 3.

**Calculation of number of PrV particles/μg and the m.o.i.**

The number of virus particles/μg was estimated using the RNA (6.4 and 2.5 kb) and protein (60 and 7.44 kDa) sizes determined for PrV in this study. The amount of protein in each particle was calculated based upon the presence of 240 copies each of the large and small capsid proteins. The molecular weight of RNA in each particle was estimated as the sum of the genomic and subgenomic RNAs. The estimated Mv values of protein and RNA in a PrV particle were calculated to be 1.62 × 107 and 0.29 × 107 Da, respectively. The estimated total particle weight (RNA plus protein) was 1.91 × 107 Da, with the RNA comprising approximately 15% of the PrV virus particle. The calculated mass of the virus particle was used to estimate the number of particles/μg of virus at 3.2 × 1010. In a typical purification, approximately 1 × 107 MG8 cells yielded about 350 μg of purified PrV (approximately 1 × 1013 particles).

**Infection of FB33 and Se-1 cells with PrV**

Cells were seeded in 35-mm diameter tissue culture plates at a density of approximately 1 × 10⁶ cells/well in 2 ml complete ExCell-401 medium (containing 10% FBS and antibiotics) and allowed to attach at 28°C. Cells were infected with 200 μl serum-free ExCell-401 medium containing 3 μg purified PrV or serial 10-fold dilutions. Mock-infected wells received 200 μl of serum-free medium only. Infections were incubated at room temperature with rocking for 2 h; then the inoculum was removed from each well, 2 ml of complete ExCell 401 medium was added, and plates were incubated at 28°C.

To analyze the production of RNA and protein over the course of infection, FB33 cells were seeded as described above and then duplicate wells were infected at an m.o.i. of approximately 1 × 10⁶ particles/cell. Duplicate mock-infected wells received 200 μl of medium only. Infected and
mock-infected wells were harvested at time points of 6, 12, 18, 24, 36, and 48 h p.i. At each time point, duplicate wells were harvested for either RNA analysis by Northern blot hybridization or protein analysis by immunoblotting (see below). In addition to time courses where RNA was detected by Northern blot hybridization, a 48-h time course where RNA was detected by metabolic labeling (as described above) was also performed. In this time course, mock and infected wells were labeled for 3 h with [3H]uridine in the presence of actinomycin D at 6, 12, 18, 24, 36, and 48 h p.i. RNAs were resolved by electrophoresis on 1% agarose-formaldehyde gels and visualized by fluorography.

Source of other tetraviruses and testing infectivity in FB33 cells

Samples of other tetraviruses were kindly supplied by Vernon Ward, University of Otago, Dunedin, New Zealand (TaV), Don Hendry, Rhodes University, Grahamstown, South Africa (NjBV), and Terry Hanzlik and Karl Gordon, CSIRO Division of Entomology, Canberra, Australia (HsSV). The infectivity of the TaV and NjBV virus stocks for their respective insect hosts could not be determined due to unavailability of the insects. The infectivity of the viral stock of HsSV was confirmed in H. armigera, the natural host (T. Hanzlik, personal communication). FB33 cells were infected or mock-infected and the RNA was metabolically labeled at 48 h p.i. as described above. Total RNA was analyzed for the presence of virus-specific bands.

Northern blot hybridization

Total cellular RNAs were extracted and resolved on 1% agarose-formaldehyde gels (3 µg/lane) as described above. RNAs were transferred to Nytran nylon membranes (Schleicher & Schuell) by capillary blotting; then the membranes were air-dried and fixed by exposure to UV light for 2 min. The two partial clones used in this study contained 705- and 544-nt inserts that were specifically labeled for parts of the PrV genome that encode nonstructural and structural proteins, respectively. Strand-specific 32P-labeled RNA probes were prepared from the T7 and SP6 promoters present in the vector using the method described previously (Albarino et al., 2001). Membranes were probed overnight at 60°C, washed, exposed to a phosphor screen overnight, and visualized using a Molecular Dynamics Storm digital radioactivity imaging system.

Western blot analysis

Total cellular proteins were harvested by adding 200 µl of 2× glycine SDS–PAGE buffer to each 35-mm-diameter plate of cells. Five microliter samples were resolved by electrophoresis on 10% glycine SDS–PAGE gels using the method of Laemmli (1970). Approximately 150 ng of purified virus alone, or mixed with 5 µl of 48 h p.i. mock-infected cells, was analyzed in parallel. Proteins were transferred to Immobilon-P membranes (Millipore) in Towbin buffer (10 mM Tris base, 96 mM glycine in 10% methanol) using a semidry graphite electroblotter (Millipore) for 2 h at a constant current of 39 mA. Following transfer, the membranes were in Ponceau S (Sigma); the position of the viral large capsid protein was marked, and the membranes destained. PrV antiserum raised in rabbits (Lampire Biologicals) against purified virus was diluted 100-fold for use as the primary antibody in the Immus-star chemiluminescent protein detection system (Bio-Rad).

TEM of purified virus and sectioned cells

Five microliters of purified virus was pipetted onto the surface of a carbon-coated copper EM grid (Electron Microscopy Sciences) and allowed to absorb for 2 min. The sample was blotted off the grid and the grid was rinsed three times with 50 mM phosphate buffer, pH 7.2, and then twice with 1% (w/v) uranyl acetate, blotting between each rinse. A final drop of 1% uranyl acetate was left on the grid for 2 min and then removed and the grid allowed to dry. Grids were examined by TEM on a Hitachi 7000.

MG8 cells (passage 100+) and infected (m.o.i. = 1 × 10³) and uninfected FB33 and Se-1 cells were harvested for cell sections and analysis by TEM. FB33 and Se-1 cells were harvested at 3 days p.i. by scraping the surface of the plate, transferring the cells and medium to microcentrifuge tubes, and pelleting by centrifugation in a microcentrifuge at 7000 rpm for 3 min. MG8 cells were scraped from a tissue culture flask and pelleted by centrifugation. Cells were rinsed in phosphate-buffered saline, repelleted, and resuspended in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 3 min and then pelleted at 5000 rpm for 3 min and left to fix for 60 min. Fixed cells were rinsed three times in 0.1 M cacodylate buffer, incubated in 1% osmium tetroxide in 0.1 M cacodylate buffer for 60 min, and then rinsed three times in cacodylate buffer followed by 10 min in 0.5% tannic acid in 0.1 M cacodylate buffer and a further three rinses with 0.1 M cacodylate buffer. The fixed cells were dehydrated by washing with a series of solutions of increasing concentrations of ethanol up to 100%. Pellets were infiltrated with Polybed 812 resin and 100% ethanol in a 1:1 proportion overnight and then in 100% Polycryl resin for three incubations of 2 h each. Pellets were then polymerized in fresh Polybed resin at 60°C overnight. Sections were collected on grids and stained in 2% uranyl acetate and in lead citrate before examination by TEM on a Hitachi 7000.

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References


