Expanding the host range of small insect RNA viruses: Providence virus (Carmotetraviridae) infects and replicates in a human tissue culture cell line

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Tetraviruses are small, positive (+ve)-sense ssRNA viruses that infect the midgut cells of lepidopteran larvae. Providence virus (PrV) is the only member of the family Carmotetraviridae (previously Tetraviridae). PrV particles exhibit the characteristic tetraviral T=4 icosahedral symmetry, but PrV is distinct from other tetraviruses with respect to genome organization and viral non-structural proteins. Currently, PrV is the only tetravirus known to infect and replicate in lepidopteran cell culture lines. In this report we demonstrate, using immunofluorescence microscopy, that PrV infects and replicates in a human tissue culture cell line (HeLa), producing infectious virus particles. We also provide evidence for PrV replication in vitro in insect, mammalian and plant cell-free systems. This study challenges the long-held view that tetraviruses have a narrow host range confined to one or a few lepidopteran species and highlights the need to consider the potential for apparently non-infectious viruses to be transferred to new hosts in the laboratory.

Tetraviruses are small, positive (+ve)-sense ssRNA insect viruses that infect the larvae of lepidopteran insects. Their name is derived from the characteristic T=4 icosahedral symmetry of their capsids, which package either a monopartite or bipartite viral genome. Tetraviruses are classified into three families according to the characteristics of their viral replicases, namely the Alphatetraviridae and Permutotetraviridae, with alpha-like and picorna-like replicases, respectively, and the Carmotetraviridae, which have a carmo-like viral replicase (Dorrington et al., 2011; ICTV Virus Taxonomy, 2011). Tetraviruses have, to date, been considered to have an extremely narrow host range confined to one or a few lepidopteran species (Dorrington & Short, 2011) infecting goblet, columnar and regenerative cells in the larval midgut (Brooks et al., 2002).

Providence virus (PrV), the only member of the Carmotetraviridae, was isolated from a persistently infected Helicoverpa zea midgut cell line and to date is the only tetravirus known to replicate in cell culture (Pringle et al., 2003). PrV particles exhibit the typical, conserved T=4 tetravirus capsid architecture (Speir et al., 2010). However, the virus is otherwise quite distinct from other tetraviruses with respect to genome organization, non-structural proteins and the regulation of viral gene expression (Walter et al., 2010). PrV has a monopartite genome of 6.4 kb encoding three ORFs (Fig. 1a). At the 5' terminus is a non-structural protein (p130), currently without assigned function. Overlapping almost entirely with the p130 ORF is the carmo-like viral replicase, p104, which is most closely related to the replicases of (+ve)-sense ssRNA plant viruses belonging to the Tombusviridae and Umbraviridae families. Similar to tombusviruses, the PrV replicase ORF encodes a Type 1 readthrough stop codon, the activity of which results in the translation of a truncated 40 kDa accessory protein, p40, at a ratio of 10:1 relative to full-length p104 (Beier & Grimm 2001; Walter et al., 2010). At the 3' end of the viral genome, just downstream of p130 and p104, is an ORF, p81, encoding the viral capsid protein precursor, which is translated from a 2.5 kb subgenomic RNA (Pringle et al., 2003; Walter et al., 2010). The p81 ORF encodes two active 2A-like processing sites at the N-terminus (Luke et al., 2008), resulting in the production of translation products of 7, 8 and 68 (p68) kDa. Virus capsids are assembled from p68 (CP), which is autoproteolytically cleaved during particle maturation into the β and γ peptides of 60 and 7.4 kDa, respectively (Pringle et al., 2003).
We used in vitro coupled transcription–translation systems to study the translation of viral gene products using a PrV cDNA clone (pFLM, GenBank accession number KX280626), which produces authentic viral RNA (vRNA) transcripts under control of the T7 promoter and a hepatis delta virus ribozyme at the 3' end of the viral cDNA sequence. We observed the presence of two \(^{35}S\)-methionine-labelled virus-specific translation products of approximately 68 and 60 kDa, corresponding in size to the PrV CP and \(\beta\) peptides, respectively, in cell-free extracts derived from insect Spodoptera frugiperda Sf21 cells (data not shown). This was confirmed by Western analysis with a rabbit anti-CP polyclonal antibody (Fig. 1b); the PrV CP was translated in cell-free extracts derived from insect S. frugi-
perda Sf21 cells containing cDNA corresponding to authen-
tic PrV genome (Fig. 1b, lane 3). CP was also translated in a wheatgerm-coupled transcription–translation system (Fig. 1b, lane 5) and less efficiently in a mammalian (rabbit reticulocyte) system charged with the PrV cDNA clone, pFLM (Fig. 1b, lane 7). The presence of the mature \(\beta\) sub-
unit, p60, in all three cell-free extracts suggested the assembly and maturation of PrV VLPs. Since the PrV capsid protein is translated from a subgenomic vRNA, we concluded that mRNA corresponding to our PrV cDNA clone must be supporting viral replication in all three in vitro cell-
free transcription–translation systems.

To determine whether viral replication was occurring, we used reverse transcription (RT) PCR to detect the presence of (−ve)-sense viral RNA in the in vitro coupled transcription and translation reactions (Promega; L1101, L5030 and L5020). The presence of (−ve)-sense vRNA was confirmed in all reactions charged with PrV cDNA by RT-PCR using either primer JRS80R (3' region of p130 ORF) or JRS78R (3' end of p81 coding sequence) for first strand syn-
thesis (Fig. 1a). RT-PCR products of 511 and 2243 bp were detected in all three reactions charged with the PrV cDNA clone, pFLM (Fig. 1c, middle panel, lanes 3, 5 and 7) but not in reactions charged with the control plasmid that does not encode any PrV sequences, pBIEX3 (Fig. 1c, middle panel, lanes 2, 4 and 6). In the RT-PCR reaction with the primers JRS77 and JRS78, targeting p81, there is a dominant 2243 kb band corresponding to the sub-genomic RNA. In addition, there is a non-specific minor band of approximately 700 bp that is present in all positive RT-PCR reactions. RT-PCR products were also detected in reactions containing pFLM when JRS79F and JRS77F were used for first strand synthesis, indicating the presence of (−ve)-sense vRNA, which could only have arisen via replication of viral mRNA transcripts. Taken together, these data suggest that the PrV cDNA clone is able to initiate vRNA replication in all three in vitro translation systems.

During the course of troubleshooting problems with the maintenance of our human cervical cancer (HeLa, ATCC number CCL-2) cell line, we noticed signs of cytopathic effect (CPE) in the cells. These included colour changes in the growth medium (DMEM) from pink to yellow after 24 h as opposed to 72 h, and increased numbers of cells that had lifted and were floating in the medium. We used immuno-
fluorescence microscopy to detect replicating dsRNA. All the cells in the culture stained positively for dsRNA, which co-localized with PrV replicase, p40/p104 in the cytoplasm (Fig. 2b). PrV capsid was also present in these cells, although the distribution of the signal did not overlap completely with that for the replicase and the dsRNA. There was no evidence of dsRNA, PrV replicase or capsid protein in HeLa cultures obtained from other groups in the depart-
ment (Fig. 2a), and we therefore concluded that our HeLa culture had become infected with PrV. Uninfected cells were inoculated with supernatant from infected HeLa cell cultures. Here, a total of 7 ml of cell supernatant from a T25 flask of infected HeLa cells exhibiting CPE was centrifuged at 2500 rpm for 10 min. The supernatant was added to a T25 flask of uninfected HeLa cells, and the cells were incubated for 1 h at 37°C in an atmosphere containing 5% CO₂. Subsequent to infection, the infected medium was replaced by fresh growth medium and the growth of the HeLa cells was resumed. After 1 week, approximately 90% of the cells stained positively for dsRNA with punctate structures distributed throughout the cytoplasm (Fig. 2c, d). While PrV replicase and capsid were also present in these cells, there appeared to be less than in the per-
sistently infected HeLa cells (compare Fig. 2c, d with Fig. 2b). Taken together, the data show that our HeLa cell culture was infected with PrV and that virus particles produced by these cells are infectious.

Virus particles were purified by differential centrifugation (Pringle et al., 2003) from different passages of persistently infected HeLa cells, taken three weeks apart. Western analy-
sis with rabbit anti-capsid polyclonal antibodies detected viral p60, representing the mature \(\beta\) capsid subunit in cell-free extracts of both the persistently infected H. zeae MG8 and HeLa cultures (Fig. 3, lanes 1–2). This protein was also detected in the virus purified from the HeLa cells (Fig. 3, ‘P1’ and ‘P2’). Since p60 is produced by assembly-
dependent maturation of tetravirus particles (Agrawal & Johnson, 1992; Gordon et al., 1999; Hanzlik et al., 1995), the data suggest the assembly of mature PrV particles in the HeLa cells. We used immuno-transmission electron microscopy (as described in Pringle et al., 2003 and Shaw et al., 2008) with the anti-PrV capsid polyclonal antibodies to confirm that the virus purified from HeLa cells was indeed PrV. The virus particles exhibited typical tetravirus surface morphology [Fig. S1 (available in the online Supplementary Material), left panel insert] and ranged in size between 18 and 58 nm with median and mode diameters of 38 nm (for \(n=200\) particles), which correlates well with the observations of Pringle et al. (2003). To confirm that these were PrV particles, we added quantum dot-labelled secondary antibodies (Invitrogen; Q11421MP) targeted to the rabbit anti-VCAP antiserum used for the Western analyses. We observed that the virus particles coincided with the electron dense quantum dots (Fig. S1, right) demonstrating that the virus particles have the epitope recognized by the anti-
VCAP antibody. Finally, we used RT-PCR to amplify the
Fig. 1. Authentic mRNA generated from PrV cDNA initiates viral RNA (vRNA) replication in vitro. (a) Schematic diagram showing the genome organization of PrV. The three ORFs are shown as p130, p104 (viral replicase) and p81 (viral capsid). The 2A-like processing sites (2A1, 2A2 and 2A3) are shown as p130 and VCAP, while the (*) in p104 indicates a readthrough stop codon. Arrows indicate the position of primer pairs used for reverse transcription (RT) PCR detection of vRNAs. (b) Detection of PrV CP (capsid precursor, p68) and β subunit (p60) in in vitro coupled transcription and translation reactions by Western analysis. For transcription, 1 µg pFLM (plasmid encoding the complete PrV genomic cDNA, NCBI accession number KX280626) or pBIEX3 (a control vector not encoding any PrV sequences supplied by Novagen) was used to charge the insect, wheatgerm and rabbit reticulocyte systems according to the manufacturer’s instructions. For Western analysis, 5, 10 and 30 µl of each reaction was resolved by SDS-PAGE and probed with rabbit anti-capsid polyclonal antibodies. MG8: PrV-infected H. zea MG8 cell extract; transcription and translation reactions charged with either plasmid pBIEX3 (−) or pFLM (+). The VCAP protein was detected after 1 s in the insect system, 10 s in the wheatgerm system and 44 s in the rabbit reticulocyte system. (c) RT detection of the (+ve)-sense and (−ve)-sense vRNA in in vitro transcription and translation coupled reactions. C, negative control (no template); −, pBIEX3; +, pFLM. RT was carried out using the QuantiTect Reverse Transcription kit (Qiagen; 205311) with primers JRS79F (CGA GGT TAC CAC AAC CTG C) or JRS77F (GCA GAA TTT ACC AGT ACC CAA TG) to detect (−ve)-sense cDNA, and JRS80R (GAT GCC TCT GGC AAC ACG CAT TCC ACC CTG TCG TCT AGC) to detect (+ve)-sense cDNA.
512 nt region at the 5' end of the p130 ORF (Fig. 1a, primer pair JRS79–JRS80) from RNA extracted from purified virus particles (miRNAeasy mini kit, Qiagen; 217004). RT-PCR reactions carried out on material prepared from uninfected HeLa cells did not yield any PCR products, leading us to conclude that the amplification of the 512 nt region was specific for PrV. This RT-PCR amplicon was ligated into the pDRIVE cloning vector (PCR cloning kit, Qiagen; 231122), and Sanger sequencing of six representative clones returned a 99% (508/512 nt) identity to the PrV isolate vFLM1 complete genome (GenBank accession number GU991616.1). Analysis of the evolutionary relationships of six clones to vFLM shows slight changes, as would be expected for a replicating RNA virus (Lauring & Andino, 2010). We plan to sequence the genome of PrV isolated from HeLa cells and that of the virus from the original MG8 cell culture line in an attempt to determine whether the virus sequence shows changes that would indicate that PrV has adapted to a new host.

The results of this study demonstrate that PrV is able to infect, replicate and produce infectious virus particles in human cancer cells. It is important to note that the ability of a virus to infect a cell line under laboratory conditions is not equivalent to the infection of an animal. It does,
however, imply that the virus particles can recognize and bind receptors on the HeLa cell surface and can enter the cells as well as initiate replication within the cytoplasm. HeLa cells, unlike Drosophila S2 cells, are not known to readily internalize unpackaged nuclear material (Ulvila et al., 2006). Whether or not the virus binds receptor(s) common to both HeLa and insect cells is unknown. The distribution of punctate structures associated with dsRNA and the viral replicase in HeLa cells is similar to what we have observed in H. zea MG8 cells, where the virus replication is associated with membranes derived from the Golgi and secretory vesicles (Short et al., 2013).

This study challenges the long-held view that tetraviruses have a narrow host range confined to one or a few lepidopteran species (Dorrington et al., 2011). Bawden et al. (1999) and Dorrington & Short (2010) hypothesized that the tetraviruses require specialized receptors found only in the lepidopteran midgut cells and/or host factors required for replication. This clearly does not apply to PrV, which is able to infect and productively replicate in HeLa cells, but this may still be true for other tetraviruses. Some members of the structurally related Nodaviridae (Banerjee et al., 2010) are known to infect both insects and vertebrates including fish and mammals (Ball et al., 1992; Johnson et al. 2004; Scherer & Hurlbut, 1967). The Betanodavirus, redspotted grouper nervous necrosis virus (RGNNV), binds but is unable to enter human cells. However, the ability of transfected RGNNV viral RNA to initiate replication in HeLa and BSRT7/5 cells suggests that this virus has the potential to infect humans (Adachi et al., 2008). The presence of nodavirus-like and dicistrovirus-like sequences in human stool samples (Finkbeiner et al., 2008; Victoria et al., 2009) has been detected by metagenomics analysis, but whether or not these viruses also replicate in insects remains to be seen.

Recently, a viral metagenomics study showed the presence of PrV (85% sequence identity) in the guano of a female western barbastelle bat (Barbastella barbastellus) in Hungary (Kemenesi et al., 2016). The presence of PrV was attributed to ingestion of infected prey since the diet of the insectivorous western barbastelle bats is predominantly lepidopteran (Zeale et al., 2011). In light of our data presented here, it is tempting to speculate that PrV was replicating in the bat rather than making a transitory journey through the animal.

PrV capsids display all the structural features of a typical tetravirus particle, although the arrangement and function of the internal helical domains appear to be more nodavirus-like (Speir et al., 2010). However, PrV is strikingly different from other tetraviruses with respect to its genome organization, non-structural proteins and the regulation of viral gene expression (Walter et al., 2010). Most notably, the carmo-like PrV replicase is most closely related to those of tombusvirus and umbraviruses, which infect only plants (Rochon et al., 2011; Ryabov et al., 2011). It is therefore not altogether surprising that we detected replication in vitro in wheat germ cell-free lysates, and we are currently investigating whether PrV is able to replicate in plant protoplasts. PrV is not the only insect RNA virus related to plant viruses. A Tylo-like virus, Culex tymbovirus-like virus (CuTLV), has been discovered in mosquitoes (Wang et al., 2012). The Tymoviridae are (+ve)-sense ssRNA plant viruses that belong to the alphavirus-like superfamily. Interestingly, there is significant conservation between both the non-structural (replicase) and structural (capsid) proteins of CuTLV and members of the Tymoviridae family suggesting a relatively recent move from plants to mosquitoes. As male mosquitoes are known to feed on plant nectar, the authors propose that this may be a route for viral transfer (Wang et al., 2012).

In a recent review, Balique et al. (2015) proposed the potential for the transfer of plant viruses to vertebrates via the food chain and discussed the potential of these viruses as human pathogens. Our study provides experimental support for this hypothesis. The tombusvirus-like characteristics of the viral replicase and evidence for replication in wheat germ cell-free extracts support a plant virus origin for the PrV replication machinery. The acquisition of an animal virus capsid, likely by recombination with a tetravirus in the lepidopteran host midgut, has resulted in PrV overcoming the natural barriers for infection of vertebrate host cells. This study highlights the need to consider the potential for apparently non-infectious viruses to be transferred to new hosts in the laboratory.

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