Virions of *Pariacoto virus* contain a minor protein translated from the second AUG codon of the capsid protein open reading frame

Karyn N. Johnson and L. Andrew Ball

Department of Microbiology, University of Alabama at Birmingham, BBRB 373/17, 845 19th St South, Birmingham, AL 35294-2170, USA

Virions of the alphanodavirus *Pariacoto virus* (PaV) have *T*=3 icosahedral symmetry and are assembled from multiple copies of a precursor protein that is cleaved into two mature capsid proteins after assembly. The crystal structure of PaV shows that the N-terminal ~30 amino acid residues of the subunits surrounding the 5-fold axes interact extensively with icosahedrally ordered regions of the encapsidated positive-sense genomic RNAs. We found that wild-type PaV particles also contain a minor capsid protein that is truncated by 24 residues at its N terminus. Reverse genetic experiments showed that translation of this protein initiated at the second AUG of the capsid protein open reading frame. When either the longer or shorter version of the capsid protein was expressed independently of the other, it assembled into virus particles and underwent maturation cleavage. Virions that lacked the shorter capsid protein retained infectivity for cultured insect cells and *Galleria mellonella* larvae.

The *Nodaviridae* are a family of small (30 nm diameter), non-enveloped, *T*=3 icosahedral viruses that primarily infect insects and fish (reviewed in Ball *et al.*, 2000; Ball & Johnson, 1998; Schneemann *et al.*, 1998). The nodavirus genome comprises two messenger-sense genomic RNAs that are co-encapsidated (Krisha & Schneemann, 1999). A non-encapsidated 0·4 kb subgenomic RNA3 is synthesized during replication. Insect nodavirus particles are assembled from the capsid protein precursor alpha, which is then autocatalytically cleaved into the two mature capsid proteins, beta and gamma (Gallagher & Rueckert, 1988; Hosur *et al.*, 1987). Schneemann *et al.* (1992) have shown that maturation cleavage of capsid protein of *Flock House virus* (FHV) is necessary for infectivity.

The genome of the insect nodavirus *Pariacoto virus* (PaV) comprises a 3·0 kb RNA1 that encodes the RNA-dependent RNA polymerase and a 1·3 kb RNA2 that encodes protein alpha (Johnson *et al.*, 2000, 2001; Zeddam *et al.*, 1999). The 401 aa protein alpha is cleaved between residues 361/362 to yield the mature capsid proteins (Johnson *et al.*, 2000; Tang *et al.*, 2001). The 3 Å crystal structure of wild-type PaV particles (Tang *et al.*, 2001) indicates that the capsid comprises 180 chemically identical protein subunits. The N termini of the 60 subunits that surround each 5-fold axis interact extensively with icosahedrally ordered regions of the encapsidated RNAs. This N-terminal region is highly basic in both PaV and other nodaviruses (Johnson *et al.*, 2000; Kaesberg *et al.*, 1990) and has been implicated in the specificity of RNA encapsidation in FHV (Dong *et al.*, 1998; Marshall & Schneemann, 2001).

We showed previously that PaV particles contained the mature capsid proteins beta and gamma and a small amount of uncleaved capsid precursor alpha. We also observed minor proteins migrating faster than beta that reacted with anti-PaV antibodies (Fig. 1 and Johnson & Ball, 2001). This suggested that not all PaV virion proteins were chemically identical, and that at least some capsids might therefore exhibit protein asymmetry. To examine this possibility, we tested whether the second AUG of the capsid protein ORF (Fig. 1B) initiates translation of an N-terminally truncated protein.

We investigated whether the additional immunoreactive proteins were related to PaV capsid proteins. Wild-type PaV particles purified from *Galleria mellonella* larvae (Johnson & Ball, 2001) were resolved by SDS-PAGE and proteins visualized by staining. The protein bands were excised from the four regions of the gel denoted A–D in Fig. 1(A), digested with trypsin and analysed by MALDI-TOF mass spectrometry (method in Johnson *et al.*, 2003). All four of the excised protein bands had tryptic peptide fingerprints characteristic of the PaV capsid protein (data not shown), confirming that each band contained proteins that were products of the capsid protein ORF. However, none of the proteins yielded an identifiable N-terminal peptide.

The N-terminal sequences of the capsid protein species in bands B and C were analysed by direct protein sequencing. Capsid proteins from PaV were resolved by SDS-PAGE, transferred to PVDF membranes in 10 mM CAPS buffer pH 11·1, 10 % methanol, for 1 h at 400 mA and stained...
Fig. 1. (A) Protein composition of wild-type PaV purified from *Galleria mellonella*. The proteins were separated by electrophoresis on SDS-10% polyacrylamide gels and visualized by staining with colloidal Coomassie blue. Lane 1 contains molecular mass markers with sizes in kDa on the left. Lane 2 contains 15 μg of wild-type clonal PaV. Gel regions A, B, C and D were excised for MALDI-TOF analysis. (B) Schematic of mutations that alter translation from the capsid ORF. The RNA sequence of the region encoding the N terminus of the capsid protein is shown with the amino acid sequence of wild-type PaV above. Mutations were introduced by site-directed mutagenesis into the RNA2 plasmid PaV2(0,0). In the 25ko plasmid a nucleotide substitution (G97A) changed the codon for Met25 to encode Ile25 and a silent mutation (G91T) was introduced to prevent initiation of translation at Val23. In the 1ko25en plasmid the first AUG of the capsid protein ORF (Met1) was mutated to ACG (T24C) to prevent initiation of translation, and a UAA stop codon was introduced two codons downstream (C30A). The translational sequence context of Met25 was enhanced by the mutation C92A. In the 1ko25ko plasmid, both the first and second methionines of the capsid protein ORF were mutated by the introduction of four substitutions (T24C, C30A, G91T and G97A). RNA2 plasmids encoding cleavage-deficient capsid proteins were made by changing Asp68 to Asn (D68N) by substituting nucleotides G224A and T226C (not shown). (C) Capsid proteins predicted from wild-type and mutant RNAs. The expected protein products for initiation of translation at Met1 and Met25 are shown with their calculated molecular masses. For wild-type and each mutant the predicted presence (+) or absence (−) of each of these products is indicated. (D) PaV capsid proteins synthesized in BSRT7/5 cells transfected with PaV cDNA clones. Cells were mock transfected (lane 2) or transfected with 2.5 μg of PaV1(1,0) and 1.0 μg of PaV2(0,0) (lane 3), D68N (lane 4), 25ko (lane 5), 25ko/D68N (lane 6), 1ko25en (lane 7), 1ko25en/D68N (lane 8) or 1ko25ko (lane 9). Cells were lysed 4 days p.t. and total cellular protein resolved on SDS-10% polyacrylamide gels. A sample of wild-type PaV purified from insects (lane 1) was included for comparison. Proteins were transferred to PVDF membranes, probed with a rabbit antiserum raised against purified PaV particles, and visualized by chemiluminescence (for method see Johnson & Ball, 2001).
with Ponceau S. Bands B and C were excised and subjected to five cycles of N-terminal sequencing. The N-terminal sequence of band B (VSRTK – with strong signals for V, S and T) was consistent with translation of the major PaV capsid protein initiating at the first AUG of the ORF (nt 23–25 of RNA2) and subsequent removal of the initiating methionine residue (Fig. 1B). The N-terminal sequence of band C was not reliable enough to unambiguously define the sequence, so we used reverse genetics to establish the origin of the protei ns.

Plasmids that express full-length infectious transcripts of RNAs 1 and 2 [called PaV1(1,0) and PaV2(0,0), respectively] have been described previously (Johnson & Ball, 2001; Johnson et al., 2000). We used site-directed mutagenesis on PaV2(0,0) to introduce mutations designed to eliminate translation from the first or second AUG codon in the capsid protein ORF, or from both codons, as shown schematically in Fig. 1(B). For clarity, we will refer to the proteins initiated at these two AUG codons as the 'Met1' and 'Met25' proteins, respectively. Translation initiation from the Met25 codon was enhanced by altering the surrounding sequence (Fig. 1B) so it was more similar to the invertebrate and vertebrate consensus sequences (Cavener & Ray, 1991). For the wild-type and each mutant the calculated sizes for the predicted Met1 and Met25 alpha and beta proteins are shown in Fig. 1(C).

BSRT7/5 cells constitutively expressing cytoplasmic T7 RNA polymerase (Buchholz et al., 1999) were transfected with plasmids PaV1(1,0) and either wild-type or mutant PaV2(0,0) plasmids. In all cases, the RNAs were confirmed to replicate by metabolic labelling in the presence of actinomycin D (data not shown). Four days post-transfection (p.t.), the cells were lysed and proteins resolved by SDS-PAGE with an aliquot of the same wild-type virus sample as shown in Fig. 1(A) for comparison. PaV-specific proteins were detected by immunoblotting using a polyclonal rabbit antisemirum raised against purified PaV (Johnson & Ball, 2001).

Wild-type RNA2 directed the synthesis of PaV-specific proteins (Fig. 1D, lane 3), which comigrated with proteins detected in PaV purified from insects (Fig. 1D, lane 1). In order of increasing mobility these corresponded to band A, which we interpreted as uncleaved Met1 alpha; band B, which we initially interpreted as the larger of its two cleavage products, Met1 beta; and a protein with the mobility of band C in Fig. 1(A). Gamma is not detected using this method. Band C had an estimated molecular mass of 36 kDa and was also detected in lysates of BSRT7/5 cells transfected with non-recombinant PaV vRNA (data not shown). Proteins corresponding to band D were sometimes detected (for example Fig. 1D, lane 1). Lysates of cells that received the 25ko plasmid contained proteins that comigrated with authentic PaV alpha and beta proteins but no detectable 36 kDa protein (Fig. 1D, lane 5), suggesting that this protein resulted from initiation at the second AUG codon and not from degradation or cleavage of the Met1 proteins. In contrast, the 1ko25en mutant produced abundant amounts of both the 36 kDa protein and a protein that comigrated with Met1 beta (Fig. 1D, lane 7). The calculated molecular mass for Met25 alpha differs from Met1 beta by only 1-5 kDa (Fig. 1C), so it was likely that the two predominant capsid proteins made by 1ko25en corresponded to Met25 alpha and beta. These data also suggest that band B seen in wild-type virus most likely contained two protein species, namely Met1 beta (39-1 kDa) and Met25 alpha (40-6 kDa), which comigrated as a single band. The lack of a clear N-terminal sequence corresponding to Met25 alpha in band B suggests that the amount of Met25 alpha in band B is low. However, the comigration of these proteins obscured their relative contributions to band B.

To examine the relationship of these proteins further, aspartic acid 68 in the capsid protein ORF was mutated to asparagine (D68N). This mutation was designed to abrogate the cleavage of alpha, because in the 3-D structure of PaV (Tang et al., 2001) the side-chain carboxylate of D68 lies close to the scissile peptide bond between beta and gamma, and occupies an equivalent position to that of residue D75 in FHV which mediates the autocatalytic cleavage of FHV alpha (Wery et al., 1994; Zlotnick et al., 1994). Accordingly, we interpret the two major bands detected in cells expressing the D68N mutant (Fig. 1D, lane 4) as the uncleaved alpha proteins from Met1 and Met25. In agreement with this interpretation, only one major capsid-specific band was detected in cells that expressed 25ko/D68N (lane 6) or 1ko25en/D68N (lane 8), and in each case the single band comigrated with the putative alpha protein directed by the corresponding cleavage-competent version. A third minor band migrating slightly faster than the Met25 beta protein was detected in cells expressing the D68N mutant (Fig. 1D, lane 4). It may represent an additional minor capsid protein or indicate that cleavage was not completely blocked by the D68N mutation. Since Met1 beta and Met25 alpha comigrate, if cleavage is not completely blocked by the D68N mutation, we cannot rule out the possibility that the D68N mutant band we interpret as Met25 alpha contains small amounts of Met1 beta. The 1ko25ko construct that had both Met1 and Met25 altered made no detectable capsid protein (Fig. 1D, lane 9). Taken together, these results indicate that PaV particles contain a minor capsid protein which is initiated from the second AUG of the capsid ORF. This protein is initiated from Met25 and is cleaved to liberate a 36 kDa beta protein (band C) that was detected in wild-type particles both by staining and immunoblot analysis.

Since cleavage of alpha occurs after nodavirus assembly (Gallagher & Rueckert, 1988), cleavage of the mutant capsid proteins observed in Fig. 1(D) suggested that both the longer and shorter versions of the capsid protein could self-assemble into virions. To produce enough virus particles for analysis we developed a two-step transfection procedure using BSRT7/5 cells which are refractive to infection with PaV, thereby reducing the possibility of selection for revertants. BSRT7/5 cells in 35 mm wells were transfected with 2-5 µg of plasmid PaV1(1,0) and 2-5 µg of either
PaV2(0,0), 1ko25en or 25ko. Two days p.t. total cellular RNA was extracted and 15% of the RNA was used to transfect a second culture of BSRT7/5 cells in 100 mm dishes. Four days p.t. the cells were lysed by addition of 0.1% NP40, the lysates clarified by centrifugation (9000 g, 15 min, 4°C), and virus was pelleted through a 30% sucrose cushion in 50 mM sodium phosphate buffer, pH 7.2 (100,000 g, 3 h, 10°C). Resuspended virus was layered onto 15–45% sucrose gradients and centrifuged for 2-25 h at 200,000 g, 10°C. The gradients were collected on a Biocomp piston gradient fractionator and the fractions containing virus particles were pooled and concentrated in Microcon YM-30 concentrators (Millipore).

Proteins that assembled into purified virus particles were resolved by SDS-PAGE and visualized by staining (Fig. 2A). Wild-type virions purified from infected larvae or from transfected BSRT7/5 cells yielded the same pattern of proteins (Fig. 2A, lanes 2 and 3). However, particles purified from BSRT7/5 cells appeared to contain about twice as much of the Met25 beta protein as those prepared in insects. Particles derived from the 25ko and 1ko25en mutant capsid proteins each contained two proteins with the mobilities expected for Met1 alpha and beta, and Met25 alpha and beta, respectively. Less cleavage of alpha was evident in 1ko25en particles compared to those assembled from the Met1 protein (Fig. 2A, lanes 5 and 4). Particle morphology was examined by electron microscopy of negatively stained preparations (Fig. 2B). Although the yields of particles recovered from both mutants were reduced relative to wild-type virus, wild-type and mutant particles were morphologically similar at this level of resolution.

Infectivity of the mutant particles was tested in FB33 cells (Johnson & Ball, 2001; Kariuki et al., 2000). FB33 cells were infected over a wide range of m.o.i.s with insect-derived PaV or with wild-type and mutant particles recovered from BSRT7/5 cells. Total cellular RNA was extracted 24 h post-infection (p.i.) and analysed on Northern blots. In all cases, both genomic segments and subgenomic RNA3 were detected (Fig. 3), indicating that the mutant virus particles were infectious in cell culture. The infectivity of the wild-type and mutant particles was within 10-fold when assayed using this method.

To determine whether the mutant virus particles could establish and maintain a productive infection, FB33 cells were infected at a m.o.i. of 30 particles per cell. Fourteen days p.i. the cells were lysed, treated with RNase A, and virus was pelleted through a 30% sucrose cushion. The mutated

**Fig. 2.** (A) Protein composition of wild-type and mutant PaV particles purified from BSRT7/5 cells. The proteins in samples purified from BSRT7/5 cells were separated by electrophoresis on SDS-10% polyacrylamide gels and visualized by staining with Coomassie blue. Lane 1 contains the molecular mass standards, with sizes on the left. Lane 2 contains wild-type particles purified from insects; all other lanes contain wild-type (lane 3), 25ko (lane 4) or 1ko25en (lane 5) particles purified from BSRT7/5 cells. (B) Electron micrographs of gradient-purified authentic and mutant particles prepared in BSRT7/5 cells. Particles were applied to the surface of a 300 mesh carbon-coated grid (Electron Microscopy Sciences) and allowed to absorb for 10 min before excess liquid was removed with filter paper. The grids were then negatively stained using 1-5% uranyl acetate solution for 35 s, stain was removed with filter paper and grids air dried. In each case the size bar represents 100 nm. The number of particles shown is not representative of the yield of particles.
regions of the 25ko and 1ko25en capsid protein genes were amplified by RT-PCR; their nucleotide sequences were determined and the introduced mutations were found to be stable. After a second passage through FB33 cells the wild-type and 25ko sequences were still unchanged, but RNA2 could no longer be detected by RT-PCR in the 1ko25en mutant sample. These results showed that the 25ko mutant could maintain a productive infection in FB33 cells with no evidence of reversion of the M25I mutation. In contrast, very little 1ko25en virus was recovered from inoculated larvae, indicating that this mutant virus was debilitated. Analysis by Krishna et al. (2003) of an analogous FHV mutant that lacked the first 31 aa of the capsid protein showed that less RNA2 was encapsidated. At lower m.o.i.s in FB33 cells less RNA2 was detected for the 1ko25en mutant as compared to wild-type PaV (Fig. 3, compare lanes 3 and 11), suggesting that particles made from the truncated PaV capsid protein may be less infectious because of a similar phenomenon.

In summary, wild-type PaV particles contain a small amount of a protein that initiates at the second AUG codon of the capsid protein ORF. Mutant particles that lacked this protein were infectious in both insect cell culture and larvae, where the mutations remained stable during passage. Evidently any selection pressure that may have existed in these experiments was insufficient to restore expression of the truncated capsid protein, suggesting that it exerts at most a subtle effect on the virus phenotype under these conditions. Although a biological role for the truncated capsid protein has yet to be identified, it may nevertheless provide an experimental opportunity to introduce structural asymmetry into the T=3 icosahedral virions for analytical or technological purposes.

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