Expression of Tobacco Ringspot Virus Capsid Protein and Satellite RNA in Insect Cells
and Three-Dimensional Structure of Tobacco Ringspot Virus-like Particles

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The capsid protein gene of tobacco ringspot virus (TobRV), which had been modified to contain an amino-terminal methionine codon, was ligated into a baculovirus transfer vector downstream from the polyhedrin promoter. The resulting plasmid was cotransfected with linearized baculovirus DNA into insect cells. Recombinant baculovirus expressed high levels of the TobRV capsid protein that assembled to form virus-like particles that were similar in size and shape to authentic TobRV capsids. These virus-like particles did not encapsidate any RNA, including the capsid protein mRNA. The capsid protein mRNA is a truncated RNA 2, which may lack a putative encapsidation signal. To determine whether an intact packaging substrate could be encapsidated by the TobRV capsid protein, another recombinant baculovirus, concomitantly expressing both capsid protein and TobRV satellite RNA, was constructed. Surprisingly, the vast majority of the satellite RNA molecules expressed from this recombinant baculovirus were ligated in the insect cells to form circular RNA molecules. Like circular forms of satellite RNA generated in planta, these circular satellite molecules remained unencapsidated by the TobRV capsid protein. Computer-generated three-dimensional reconstruction using electron cryomicrographs of the empty virus-like particles allowed the first structural analyses of any nepovirus capsid. This 22-Å resolution reconstruction resembled capsids of other members of the picornavirus superfamily. These data support the hypothesis that the nepovirus capsid is structurally analogous to those of the como- and picornaviruses.

INTRODUCTION

Tobacco ringspot virus (TobRV) is a member of the nepovirus group. The TobRV genome consists of two plus-sense molecules of linear, single-stranded RNA. Both of these RNAs are translated as polyproteins, which subsequently are processed to the final virus gene products (Jobling and Wood, 1985; Forster and Morris-Krsinich, 1985). RNA 1 is approx 7500 nucleotides (nt) (Diener and Schneider, 1966; Rezaian and Francki, 1974), and by analogy with other nepoviruses (Greif et al., 1988; Ritzenthaler et al., 1991), is thought to encode proteins required for virus replication. RNA 2 is approx 3900 nt and encodes the 57-kDa capsid protein in its 3′-terminal region (Buckley et al., 1993, 1995). A putative movement protein is likely to be encoded in the 5′ region of RNA 2 as has been demonstrated for the closely related tomato ringspot nepovirus (Wieczorek and Sanfacon, 1993).

The icosahedral TobRV capsids may contain a single molecule of RNA 1, two molecules of RNA 2, or a single molecule of RNA 2 (Diener and Schneider, 1966). Empty TobRV capsids, which sediment as a separate virus component, readily form as evidenced by their abundance in TobRV preparations (Stace-Smith et al., 1965). In some TobRV infections, a 359-nt satellite RNA also is encapsidated by the TobRV capsid protein (Schneider and White, 1976). Rolling circle replication has been proposed for the satellite RNA (Kiefer et al., 1982), based primarily upon the presence of linear, multimeric, and circular forms of both polarities in tissues from infected plants. Satellite RNA propagation is dependent upon the supporting TobRV, although any specific details of the propagative cycle for satellite RNA are largely speculative at this point. Only linear forms of the satellite RNA molecules are encapsidated (Linthorst and Kaper, 1984; Bruening et al., 1991).

The supporting TobRV provides the capsid protein for encapsidation of the satellite RNA (Schneider and White, 1976). The mechanism by which the satellite usurps the TobRV capsid protein is unknown, as it shares no sequence similarity with the TobRV genome (Rezaian and Jackson, 1981). Indeed, the mechanism by which the TobRV capsid protein is able to recognize and package specifically each of its own genomic RNAs, to the exclusion of other nucleic acids, is unknown. The recognition signal for packaging may be a specific sequence or a secondary structural feature present in the RNA that interacts with a capsid protein subunit, oligomer, or capsid. As the satellite RNA does not share any sequence similarity with the TobRV RNAs, any putative encapsidation signal is more likely to be a secondary structural feature than a primary sequence. In addition to the capsid protein, other virus-
or host-encoded factors also may play a role in recognizing the RNA to be packaged.

The capsid appears to be the same for all TobRV and satellite components in terms of both size and shape (Schneider, 1969). However, no atomic resolution structure has been solved for any nepovirus. The molecular weight of an empty capsid has been reported as 3.4 × 10^6 Da (Heuss et al., 1981). Given that the molecular weight of the capsid protein is 57 kDa, a T = 1 structure composed of 60 subunits is predicted (Buckley et al., 1993). A secondary structure analysis of the capsid protein predicted that there are 3 β-barrel domains per capsid protein subunit. For this reason, we have suggested that the TobRV capsid is structurally analogous to the comoviruses and picornaviruses (Lomonossoff and Johnson, 1993) and that a pseudo T = 3 designation containing 180 β-barrels may be more appropriate (Buckley et al., 1993).

Toward understanding the structural and functional properties of the TobRV capsid protein, recombinant baculovirus either expressing the TobRV capsid protein alone or concomitantly expressing the TobRV capsid protein and the satellite RNA were generated. Results of TobRV capsid assembly and RNA encapsidation studies utilizing insect cells infected with these recombinant baculoviruses, as well as the first structural analyses of any nepovirus capsid, are presented.

**MATERIALS AND METHODS**

**Generation of recombinant baculovirus**

The TobRV capsid protein gene, which previously had been modified to contain an amino-terminal methionine codon (Buckley et al., 1993), was excised from pSSSCP-2.1 (Buckley et al., 1993) with BamHI and was ligated to a baculovirus transfer vector, pVL941 (PharMingen), that had been linearized with BamHI. The resulting plasmid, which contained the capsid protein gene downstream from the polyhedrin promoter, was used to transform Escherichia coli, DH5αF′IQ (Gibco BRL). Recombinants were picked using ampicillin selection and screened for the presence of the capsid protein gene by colony blot hybridization using a 32P-labeled, satellite riboprobe. Plasmid p20SD(-3) (Ponz et al., 1987) was linearized with HindIII and transcribed with T7 RNA polymerase in the presence of [α-32P]CTP to generate minus-strand satellite RNA riboprobe. Plasmid DNA was prepared from hybridization blot-positive colonies and digested with HincII and HindIII to determine the orientation of the satellite cDNA. Clone pSat10 contained the dimeric satellite cDNA in the orientation such that transcription from the p10 promoter would yield plus-strand satellite RNA.

Plasmid pSat10 contained a BamHI site in the EcoRI satellite insert and another BamHI site downstream from the polyhedrin promoter. For this reason, pSat10 was partially digested with BamHI, and full-length pSat10 molecules that had been linearized by BamHI were separated from other fragments from the digest by gel electrophoresis. These were ligated to the TobRV capsid protein gene that had been excised from pSSSCP-2.1 (Buckley et al., 1993) using BamHI. The resulting plasmid, pSat10CPph, was amplified and screened using 32P-labeled, capsid protein gene riboprobe as described above. DNA was prepared from blot-positive colonies and digested with SphI and HindIII to ascertain that the capsid protein gene was downstream from the polyhedrin promoter and in the proper orientation. Plasmid pSat10CPph, which contained the circularly permuted, dimeric satellite cDNA downstream from the p10 promoter and the TobRV capsid protein gene downstream from the polyhedrin promoter, was used to generate a recombinant baculovirus clone, AcSat10CPph, as described above.

**Expression of capsid protein and satellite RNA in baculovirus-infected SF9 cells**

AcCPph or AcMNPV (wild-type) baculovirus were used to inoculate 1.8 × 10^8 SF9 cells at a multiplicity of infection.
(m.o.i.) of 6 in a spinner flask in Hink's media containing 0.5% fetal bovine serum and 1× pluronics (Gibco). At 5 days p.i., a 10-ml sample was removed from the culture and the cells were separated from the media by centrifugation at 500 g. The cell pellet was resuspended in 0.5 mL of 20 mM NaHPO₄, adjusted to 2.5% SDS and 350 mM 2-mercaptoethanol. A sample containing 6 μg of total cellular protein was heated to 100°C for 30 sec, analyzed by SDS–PAGE (Laemmli, 1970), and visualized by silver staining (Sammons et al., 1981).

For the time course of capsid protein expression, AcSat₀₀CPₚₚ was used to inoculate 1 × 10⁶ Sf9 cells per 35-mm plate in Hink's media at a m.o.i. of 10. Each day, for 6 days, nascent cellular proteins were labeled with [³⁵S]methionine as previously described (O'Reily et al., 1992). The medium was collected and the cells were rinsed twice with ice-cold phosphate-buffered saline. The medium plus the two washes were concentrated by ultrafiltration using Centricon 30 (Amicon) as per the manufacturer's instructions. The retentate (100 μl) was adjusted to 3% glycerol, 0.23 M 2-mercaptoethanol, and 1% SDS and a 50-μl sample was analyzed by SDS–10% PAGE and visualized by autoradiography.

To monitor accumulation of satellite RNA in infected insect cells, AcSat₀₀CPₚₚ was used to inoculate 2 × 10⁸ Sf9 cells in 100 ml of Hink's media at a m.o.i. of 3 as described above. A 5-ml sample was removed before inoculation and then each day for 6 days. Cells were separated from the media by centrifugation at 500 g. The cell pellet was resuspended in 10 ml of lysis buffer (10 mM KCl, 1.5 mM magnesium acetate, 20 mM HEPES, pH 7.4, 2.5 mM dithiothreitol), frozen at −70°C, thawed, and then disrupted using a Dounce homogenizer. The nuclei were pelleted by centrifugation at 11.2 K for 10 min and then resuspended in 10 ml of lysis buffer. The supernatant liquid containing the cytoplasm and the resuspended nuclei each were extracted using an equal volume of phenol:chloroform:isoamyl alcohol (50:49:1) and precipitated using 0.3 M sodium acetate and ethanol. Nuclear and cytoplasmic samples were resuspended in water, treated with RNase-free DNase (Promega), separated on an 8% acrylamide (acrylamide: bis, 38:2)/7 M urea gel, and blotted to a Zetaprobe membrane (Bio-Rad). Blots were hybridized with ³²P-labeled, minus-strand satellite riboprobes, visualized by autoradiography, and beta radiation-emitting bands were quantified on a Betascope (Model 603) blot analyzer.

Purification of TobRV-like particles from insect cells

AcCPₚₚ was used to inoculate 8 × 10⁸ Sf9 cells at a m.o.i. of 3 as described above. Six days p.i., cells and cellular debris were separated from the media by centrifugation at 45 g for 10 min. The cell pellet was extracted with chloroform:butanol (1:1) and the aqueous phase was loaded onto a 30% sucrose cushion and spun at 260K g for 1.5 hr at 4°C. The resulting pellet was resuspended in 20 mM NaHPO₄. The medium was loaded onto a 30% sucrose cushion and spun at 260K g for 1.5 hr at 4°C. The pellets were resuspended in 20 mM NaHPO₄.

For the time course of virus-like particle accumulation, AcCPₚₚ was used to inoculate 1 × 10⁶ Sf9 cells in 60 ml of Hink’s media at a m.o.i. of 3 as described above. A 2.5-ml sample was removed each day for 6 days. Cell viability was monitored daily using a 0.2% solution of the exclusion dye, trypan blue. Cells were separated from the media and processed as described above.

For preparative purification of virus-like particles, cells and cellular debris were removed by centrifugation at 12.4K g for 10 min at 4°C. The supernatant medium was loaded onto a 30% sucrose cushion and was spun at 140K g for 2 hr at 4°C. The pellet was resuspended in 20 mM NaHPO₄, extracted with an equal amount of butanol:chloroform (1:1), and CsCl was added to a concentration of 0.43 g/mL. A gradient was established by centrifugation at 147K g for 18–20 hr at 22°C. Following gradient fractionation, a sample of each fraction was loaded onto an SDS–10% polyacrylamide gel. Proteins were separated by electrophoresis and visualized by silver staining. The fractions containing the 57-kDa capsid protein were pooled, diluted sixfold with 20 mM NaHPO₄, and pelleted at 260K g for 1.5 hr at 22°C to remove the CsCl. This pellet was resuspended in 20 mM NaHPO₄ and purified further using a second CsCl density gradient. The amount of purified capsid protein was measured by the method of Bradford (1976) (Bio-Rad) using an albumin standard.

Samples containing the preparatively purified 57-kDa TobRV-like particles were loaded onto 400-mesh carbon–collodion-coated copper grids. The grids were washed with distilled water, stained with 1% aqueous uranyl acetate, and examined at room temperature with an 80-kV potential using a CM10 transmission electron microscope. The images were recorded at 39K× magnification.

To extract any nucleic acids contained within the virus-like particles, a 35-μg sample of CsCl-purified virus-like particles was adjusted to 1.5% SDS and extracted with an equal volume of phenol:chloroform:isoamyl alcohol (50:49:1). The aqueous phase was adjusted to 0.3 M sodium acetate and precipitated with ethanol.

Separation of TobRV components

Satellite-free TobRV was propagated in Black Valentine bean plants (Phaseolus vulgaris) and prepared as described previously (Buckley and Bruening, 1990). The virus suspension was loaded onto a 30% sucrose cushion and spun at 230K g for 2 hr at 4°C. The pellet was resuspended in 20 mM NaHPO₄, and CsCl was added to a concentration of 0.43 g/ml for separation of top component or 0.63 g/ml for separation of middle and bottom components. A gradient was formed by room-tempera-
ture centrifugation at 288 K for 18 hr for separation of top component and 147 K for 20 hr for separation of middle and bottom components. The gradients were fractionated. The refractive indices of the fractions from the CsCl purification of the TobRV components (and virus-like particles) were measured using a Bausch & Lomb Abbe-3L refractometer that had been standardized by the method of Bruner and Vinograd (1965). Conversion of the refractive indices to density was made using the International Critical Tables. Fractions containing top, middle, or bottom components were pooled separately, diluted at least sixfold in 20 mM NaHPO₄, and pelleted at 210K–230K g for 1.5 hr. The pellets were resuspended and the RNA was extracted from each component as described above for virus-like particles.

Preparation of total RNA

AcSat₂₀CPₘₙ was used to inoculate 1 × 10⁸ Sf9 cells in 90 ml of Hink’s media at a m.o.i. of 6 as described above. A 10-ml sample was removed 4 days p.i. Cells were separated from the media by centrifugation at 500 g. The cells were resuspended in 5 ml of guanidine thiocyanate lysis buffer (4 M guanidine thiocyanate, 0.1 M Tris–HCl, pH 7.5, 0.14 M NaHPO₄, and 0.5% sarkosyl), extracted using an equal volume of phenol:chloroform:isoamyl alcohol (50:49:1), and precipitated with 0.3 M sodium acetate and ethanol. Nucleic acids were resuspended in 700 µl of water. A 3-µl sample was treated with 1 U of RNase-free DNase (Promega), separated on an 8% acrylamide (acrylamide:bis, 38:2)/7 M urea gel.

To prepare total RNA from satellite/TobRV-infected plant tissues, a 2-g sample of bean plant (P. vulgaris var. Black Valentine) leaf tissue that had been infected with a preparation of TobRV and satellite RNA was harvested 24 days p.i. Total RNA was purified by a method similar to that described by Slater (1984), except that leaves initially were ground in a mixture of 10 ml of guanidine thiocyanate lysis buffer (see above) plus 10 ml of the detergent mix. A 120-µg sample of total nucleic acids, in 500 µl of water, was treated with 50 U of RNase-free DNase. Following phenol:chloroform extraction and ethanol precipitation, a 1-µg sample was separated on an 8% acrylamide/7 M urea gel.

Electron cryomicroscopy and three-dimensional computer reconstruction

Five-microliter samples of TobRV-like particles containing 4 mg/ml of protein were applied to electron microscopy grids and embedded in vitreous ice using established procedures (Adrian et al., 1984; Dubochet et al., 1988; Prasad et al., 1994). Electron cryomicroscopy was carried out at −155° with a 100-kV potential using a JEOL 1200 electron microscope. Images were recorded at 30K× magnification.

Electron micrographs without visible drift were chosen for computer image processing. The micrographs were digitized on a Perkin–Elmer microdensitometer with a step size of 16 × 16 µm, which corresponded to 5.33 Å in the object. The individual particles were boxed in a 128 × 128 pixel area and masked with an appropriate radius. The three-dimensional reconstruction was carried out as described previously (Crowther, 1971; Fuller, 1987; Prasad et al., 1990, 1994; Baker et al., 1991). The three-dimensional structure of TobRV-like particles initially was determined from three sets of 20 particles selected from the micrograph recorded at 1.3 µm under focus. The 20 best particles from the three sets, which had phase residuals less than 50° and had unique orientations in the asymmetric unit of the icosahedron, were chosen for the final reconstruction. All of the computations were performed on a Unix-based Silicon Graphics workstation and the surface representations of the three-dimensional density maps were displayed using IRIS Explorer (Silicon Graphics Inc.). The radial density plot was computed from the final three-dimensional map by averaging densities in concentric shells of 5.33 Å width.

RESULTS

Expression of the TobRV capsid protein in insect cells

The TobRV capsid protein gene, which previously had been modified to contain an amino-terminal methionine codon (Buckley et al., 1993), was ligated into a baculovirus transfer vector, downstream from the polyhedrin promoter (Fig. 1A). The resulting plasmid, pCPₘₙ, was used to generate recombinant baculovirus. One of the recombinants, AcCPₘₙ, containing the capsid protein gene, was amplified and tested for its ability to direct the synthesis of the TobRV capsid protein in Sf9 insect cells.

Total proteins from AcCPₘₙ-infected cells were examined for the presence of the capsid protein. As seen in Fig. 2, lane 1, a prominent band was seen at 57 kDa that comigrated with authentic TobRV capsid protein (Fig. 2, lane 2, a prominent band was seen at 57 kDa that comigrated with authentic TobRV capsid protein (Fig. 2, lane 3, a prominent band was seen at 57 kDa that comigrated with authentic TobRV capsid protein (Fig. 2, lane 4). DNase. Following phenol:chloroform extraction and ethanol precipitation, a 1-µg sample was separated on an 8% acrylamide/7 M urea gel.

Properties of baculovirus-expressed TobRV-like particles

To determine whether the baculovirus-expressed TobRV capsid protein had assembled into virus-like particles in the insect cells, a modification of the chloroform:butanol extraction method routinely used to extract TobRV from infected plant tissues (Buckley and Bruening, 1990) was performed on the insect cells and the final pellet was negatively stained and examined by electron microscopy. Numerous virus-like particles were seen from these crude preparations from both the cells and the media (data not shown).

The optimal time to harvest the AcCPₘₙ-infected insect
FIG. 1. (A) Map of pCP_ph. The TobRV capsid protein gene (CP) was ligated downstream of the polyhedrin promoter (ph) in baculovirus transfer vector, pVL941. The ampicillin resistance gene (Amp) and BamHI restriction sites are noted. (B) Map of pSat10CP_ph. Circularly permuted, dimeric satellite cDNA (Sat) was ligated into baculovirus transfer vector, pAcUW51, downstream from the p10 promoter. The TobRV capsid protein gene (CP) was ligated downstream of the polyhedrin promoter (ph). The ampicillin resistance gene (Amp) and the EcoRI and BamHI restriction sites are noted. (C) Predicted p10 transcripts and ribozyme-processed products. The primary satellite transcript from the p10 promoter is predicted to be a circularly permuted, dimeric satellite RNA containing 75 additional nt at the 5'-terminus and 67 nt at the 3'-terminus, from the vector. The satellite cDNA sequences (between the two asterisks) and the SV40 transcriptional termination signal (∗) are indicated. The numbers in parentheses indicate the nt number of positive-stranded satellite RNA. This primary transcript will self-process due to its inherent ribozyme activity to yield an authentic, 359-nt satellite RNA, complete with 5'-hydroxyl and 2',3'-cyclic phosphate termini and the 5'- and 3'-terminal cleavage by-products.

cells for maximal yields of virus-like particles was determined by monitoring daily accumulation of virus-like particles from cells and media for 6 days following inoculation. Capsid protein accumulation was detected as early as 1 day p.i. from both cells (data not shown) and media (Fig. 3, lane 4), but as shown in Fig. 3, lane 8, the largest accumulation of the virus-like particles was found in the media at 5 days p.i.

The virus-like particles subsequently were purified preparatively from the media portion of the infected cells at 5 days p.i. and were further purified utilizing CsCl density gradients. As shown in Fig. 4, lane 4, this yielded a preparation of virus-like particles devoid of other contaminants. Using a m.o.i. of 3, a typical yield from a 400-ml spinner culture was 0.15 mg of TobRV-like particles. Larger yields were obtained using higher m.o.i.: 0.88 mg (m.o.i. 4) and 1.9 mg (m.o.i. 6). Electron microscopy of this material, which had been negatively stained with uranyl acetate, revealed icosahedral virus-like particles (data not shown).

The density of the virus-like particles as measured in CsCl was 1.285 g/ml. As shown in Table 1, this density was less than that measured for either the middle or bottom components and similar to the density measured for the top component of authentic TobRV. As these results suggested that the particles may be empty, CsCl-purified virus-like particles were extracted with phenol-chloroform–isoamyl alcohol to recover any nucleic acids contained within. No nucleic acids were detected spec-

FIG. 2. Expression of TobRV capsid protein in Sf9 insect cells. ACCP_ph and AcMNPV (wild-type) baculovirus were used to infect Sf9 cells. At 5 days p.i., the cells were separated from the media. Total protein from the cells was analyzed by SDS–10% PAGE and silver staining. Four micrograms of authentic TobRV capsid protein (CP) was loaded in lane 1. A 6-μg sample of total protein from AccP_ph-infected (lane 2), AcMNPV-infected (lane 3), or uninfected (lane 4) insect cells were loaded. Lane 5 contains molecular weight markers (MW). The mobility of the 57-kDa TobRV capsid protein is marked.

FIG. 3. Time course of TobRV-like particle accumulation. A spinner culture of Sf9 insect cells was inoculated with AccP_ph at a m.o.i. of 3. A 2.5-ml sample was removed preinoculation (lane 2) and each day for 6 days p.i. (lanes 3-9). TobRV-like particles were purified from the media portion of the culture as described under Materials and Methods and analyzed by SDS–10% PAGE and silver staining. Lanes 1 and 10 contain authentic TobRV capsid protein (CP) and molecular weight markers (MW), respectively.
by virtue of ribozyme activity to yield plus-stranded satellite RNA (see Fig. 1C). This self-processing ribozyme reaction of the circularly permuted, dimeric satellite RNA previously has been shown to generate the 5'-hydroxyl and 2',3'-cyclic phosphate termini of authentic satellite RNA (Prody et al., 1986). T7 RNA polymerase transcripts generated in vitro have been demonstrated to be infectious in plants (Gerlach et al., 1986).

TobRV capsid protein expressed in AcSat10CPph-infected cells assembled into virus-like particles indistinguishable from the virus-like particles purified from AcCPph-infected insect cells. Extraction of these particles and hybridization blot analyses using either 32P-labeled riboprobe indicated that they did not contain nucleic acid (data not shown).

Several experiments subsequently were performed to investigate the reason for the failure of the capsid protein to encapsidate the satellite RNA. Time courses to measure satellite RNA accumulation and capsid protein expression were performed to test whether the satellite RNA and the capsid protein were concomitantly present in the cytoplasm of infected insect cells such that nucleic acid packaging might occur. Results from the capsid expression time course indicated the capsid protein was maximally expressed in the SF9 cells during 4–6 days p.i. (data not shown). Similarly, satellite RNA accumulated in both the nucleus and the cytoplasm between 3 and 5 days p.i. (data not shown).

Surprisingly, the vast majority of the satellite that was detected in total RNA from AcSat10CPph-infected SF9 cells comigrated with the circular form of the satellite RNA from infected plant tissues on an 8.0% polyacrylamide/7 M urea gel (Fig. 5A, lane 1). The mobility of circular RNA molecules is greatly reduced compared to that of linear RNA molecules of the same molecular weight on this highly crosslinked polyacrylamide gel. Like authentic circular satellite RNA molecules from infected plant tissues, this satellite RNA comigrated with linear monomeric sat-

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<th>TABLE 1</th>
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<tr>
<td><strong>Densities and Nucleic Acid Content of TobRV Components and TobRV-like Particles</strong></td>
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<tr>
<td><strong>Density (g/ml)</strong></td>
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<tr>
<td>TobRV-like particles</td>
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<tr>
<td>TobRV — top component&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>TobRV — middle component&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>TobRV — bottom component&lt;sup&gt;c&lt;/sup&gt;</td>
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<sup>a</sup> Nucleic acid content from 35-μg capsid protein.
<sup>b</sup> Top component (contains no RNA 1 or 2).
<sup>c</sup> Middle component (contains one molecule of RNA 2).
<sup>d</sup> Bottom component (contains either one molecule of RNA 1 or two molecules of RNA 2).
of the star did not interconnect or lead to the interior of insect cells suggests either that the ligation reaction was dimensional structure of TobRV-like particles along the packaging by the TobRV capsid protein. Further experi-
capsid surface. These projections were approx 65 Å wide. The vast predominance of the circular forms of satellite particles were chosen for three-dimensional reconstruc-
tions are recovered in preparations of flockhouse virus, and linear forms were generated. Circular forms of satel-
ite RNA are not encapsidated in planta.

Electron cryomicroscopy and three-dimensional reconstruction

The baculovirus expression system provided sufficient yields of purified TobRV-like particles for electron cryomi-
scopy studies. An electron cryomicrograph of the TobRV-like particles is shown in Fig. 6A. These particles appeared similar in size and shape to the particles in the bottom component of authentic TobRV (Fig. 6A, inset), except that the interior of the authentic particles were electron-dense due to the encapsidated RNA. The virus-like particles were empty spherical shells with a smooth outer surface. The capsids had a diameter of approx 300 Å. The capsid diameter determination from a cryomicro-
graph of the bottom component of authentic TobRV showed a similar diameter of approx 300 Å.

Sixty particles on the micrograph initially were ana-
lyzed by computer image processing. Twenty of these particles were chosen for three-dimensional reconstruc-
tion to a resolution of 22 Å. Stereo views of the three-
dimensional structure of TobRV-like particles along the five-, three-, and twofold axes of symmetry are shown in Fig. 7. The star-shaped projections at the fivefold axes of symmetry were the most prominent features on the capsid surface. These projections were approx 65 Å wide and approx 20 Å high. The five alcoves between the arms of the star did not interconnect or lead to the interior of the capsid. The threefold axes of symmetry contained triangular plateaus with three small peaks that sloped to valleys at the twofold axes of symmetry. The valleys were approx 75 Å wide and 20 Å deep.

The radial density profile (Fig. 8) shows the distribution of the mass density of the three-dimensional structure of TobRV-like particles as a function of the radius of the capsid structure. The profile indicated that the mass density of the particle was located in a single peak between approx 95 and 150 Å from the center of the particle, which corresponded to the capsid shell. The radial density plot also indicated that there was no significant mass density inside the TobRV-like particle, confirming that the particles were empty. Mass density calculations (assuming protein density of 1.28 g/ml) indicated there were 60 molecules of capsid protein per capsid. This number is in agreement with previous estimates from an electron microscopy study (Roberts, 1988) and from the number of molecules estimated from our reported molecular weight of the capsid protein (Buckley et al., 1995) and the previously determined molecular weight of empty capsids (Heuss et al., 1981).

DISCUSSION

Formation of TobRV-like particles in insect cells demon-
strated that virus-encoded proteins other than capsid protein were not required for TobRV capsid assembly. However, despite the concomitant presence of satellite RNA (and the truncated RNA 2) and capsid protein within the cytoplasm, no RNA was encapsidated. Similar results have been reported for baculovirus-expressed virus-like particles from another nepovirus, arabis mosaic virus, although the method by which these particles were determined to be empty was not clear (Bertioli et al., 1991). These results contrast with results from similar experiments performed with flockhouse virus (Schneemann et al., 1993), where the truncated RNA encoding the capsid protein was encapsidated. Unlike TobRV, no empty parti-
cles are recovered in preparations of flockhouse virus, presumably because the RNA serves as an integral part of the capsid quaternary structure (Fisher and Johnson, 1993). These data indicate that there may be important differences in the manner in which these two viruses encapsidate RNA. In these experiments the satellite RNA molecules presumably were removed from their normal mode of replication, which may provide directions for encapsidation by the TobRV capsid protein. The repli-
case or other ancillary proteins may be required for RNA packaging by the TobRV capsid protein. Further experi-
ments are underway in plant protoplasts to determine whether other host and/or TobRV-encoded factors are required for RNA encapsidation.

The vast predominance of the circular forms of satellite RNA relative to the linear forms of satellite RNA in the insect cells suggests either that the ligation reaction was
extremely efficient or that linear satellite RNA molecules were rapidly degraded in the insect cells. Circularization by the cell may be the end result for a satellite RNA molecule that is not encapsidated. This does not imply that these circular satellite molecules are inert. Indeed, infectivity of the circular form of satellite previously has been demonstrated (Buzayan et al., 1995). However, our observation in insect cells is consistent with the observation that in planta there are more plus-stranded circular satellite RNA molecules than would seem to be required to serve as templates for the replication of the relatively small proportion of minus-strand satellite RNA molecules found in an infection (our unpublished observations; Passmore and Bruening, 1993).

The ribozyme activity inherent in the minus-strand satellite RNA readily undergoes a reversible ligation/cleavage reaction in vitro, in which linear and circular forms of satellite RNA interconvert (Buzayan et al., 1986). The

FIG. 7. Three-dimensional structure of TobRV-like particles at 22 Å. Stereo pairs of the three-dimensional reconstruction of TobRV-like particles viewed along the (A) twofold, (B) threefold, and (C) fivefold axes of symmetry are depicted.

FIG. 8. Radial density plot. A radial density profile was calculated from the three-dimensional structure of TobRV-like particles. The average density (arbitrary units) was plotted as a function of the radius (Å) of the capsid. The peak, extending from 95 to 150 Å, corresponds to the capsid shell. The negative peaks at 95 and 150 Å are due to Fresnel fringes that arise due to defocusing during recording of the electron micrograph.
circularly permuted, dimeric plus-stranded satellite RNA (transcribed from the p10 promoter in these experiments) readily self-cleaves in vitro (Gerlach et al., 1986) and in vivo (these experiments), but unlike the monomeric minus-strand satellite RNA, it does not readily autocatalytically ligate in vitro. The circular forms of satellite RNA found in planta are postulated to be generated by a plant ligase (Passmore and Bruening, 1993; Buzayan et al., 1995). The formation of circular satellite RNA molecules in these insect cells indicates that a ligase with similar substrate specificity is likely to be present in both insect and plant cells. Enzymes capable of ligating 2',3'-cyclic phosphate and 5'-hydroxyl termini to form a 2'-phospho-

The most exciting aspect of these studies was the capsid structural data. This is the first report describing the structural features of any nepovirus. The diameter and morphological features of the TobRV-like particles are similar to those of other members of the picornavirus superfamily. The diameter of authentic TobRV particles previously had been estimated from a negatively stained electron micrograph to be 280 Å (Corbett and Roberts, 1962). Our estimate of the diameter of the authentic particles from the cryomicrograph was approx 300 Å. Size determination from a cryomicrograph is more accurate, because negative staining causes approx 30% specimen shrinkage (Berriman and Leonard, 1986; Olson and Baker, 1989).

Of the icosahedral picornavirus capsid structures determined to date, all have star-shaped features at the fivefold axes of symmetry, plateaus at the threefold axes of symmetry, and valleys at the twofold axes of symmetry (Filman et al., 1985; Rossmann et al., 1985; Luo et al., 1987; Chen et al., 1989; Lomonoosoff and Johnson, 1991). The "canyons" (Rossman, 1989) surrounding the star-shaped structures on the capsids of polio-, mengo-, and rhinoviruses appear to be absent on TobRV-like particles. TobRV-like particles contain the "wall" at the northern rim of the canyon, but like the comoviruses, lack the "wall" at the southern rim. The prominence of the star-shaped protrusions at the fivefold axes of symmetry, and the lack of the southern wall, gives the virus a topographical appearance that is most similar to that of bean pod mottle virus and cowpea mosaic virus.

Based on capsid protein secondary structure analyses, we have hypothesized previously that the TobRV capsid protein is folded into three β-barrel domains, which remain uncleaved as a single polypeptide chain (Buckley et al., 1993), but are analogous to the 3 separate β-

barrels of the animal picornavirus superfamily capsid proteins and the 2 plus 1 β-barrels of the comovirus capsid proteins. The capsids of each of these viruses contain 60 copies of each capsid protein for a total of 180 β-barrels. The structural similarity between TobRV-like particles and other isometric members of the picornavirus superfamily is consistent with our hypothesis. An atomic resolution structure will be necessary to confirm the presence of the three β-barrel domains in the single capsid protein of TobRV. We currently are attempting to crystallize the TobRV-like particles for X-ray diffraction studies.

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REFERENCES


