Characterization of a Satellite RNA Associated with Pea Enation Mosaic Virus

By S. A. DEMLER AND G. A. DE ZOETEN*

Department of Plant Pathology, University of Wisconsin, Madison, Wisconsin 53706, U.S.A.

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SUMMARY

The bipartite genome of pea enation mosaic virus (PEMV) is often accompanied by a non-essential third RNA ($M_r 0.3 \times 10^6$) of unknown origin and function. Although the Wisconsin strains of PEMV originally lacked this RNA, we have monitored the appearance of a putative replicative form of this species in PEMV-infected tissue. In later generations encapsidated single-stranded RNA 3 appeared. We have used a 750 bp clone generated against the ds replicative form of RNA 3 to probe viral and host-derived nucleic acids to establish the relationship of this RNA to PEMV infection. Northern blot analysis showed that RNA 3 is distinct from viral genomic RNA and from host RNA. Similarly, Southern blot analysis showed that RNA 3 is distinct from the host genome. Infectivity analysis of fractionated viral RNAs coupled with Northern blot analysis confirmed that RNA 3 is both non-essential for PEMV infection, and non-infectious when inoculated on its own. RNA 3 does not influence symptom expression, aphid transmission or particle morphology. We conclude that RNA 3 of PEMV is a satellite RNA.

INTRODUCTION

The genome of pea enation mosaic virus (PEMV) requires two single-stranded RNAs of approx. $M_r 1.7 \times 10^6$ and $1.4 \times 10^6$ for infectivity. Many strains also contain a third encapsidated RNA of $M_r 0.3 \times 10^6$ whose role in infection has not yet been determined. Infectivity studies by Gonsalves & Shepherd (1972) and by Hull & Lane (1973) have suggested that RNA 3 is non-infectious and unnecessary for infection. Neither group assigned a definitive phenotype to this RNA, or established a relationship between RNA 3 and the two genomic RNAs. The Wisconsin strains of PEMV have, until recently, contained little or no encapsidated RNA 3. German & de Zoeten (1975) found that the abundance of encapsidated RNA 3 correlated with the use of heat in the RNA isolation procedure, and that low temperature extraction procedures yielded no RNA 3. In the same study, dsRNA analysis of PEMV-infected tissue showed an $M_r 0.7 \times 10^6$ dsRNA appropriate for the replicative form (RF) of RNA 3. Again, the abundance of this dsRNA seemed to be related to the breakdown of the RF of RNA 1 induced by alternate freezing and thawing of dsRNA preparations. It was concluded that RNA 3 was, most likely, an artefact produced by decomposition of RNA 1. A more recent dsRNA analysis has demonstrated, however, that the levels of a putative RF of RNA 3 increased with repeated host passage, eventually becoming the dominant dsRNA in infected tissue. Initially, this increase in dsRNA 3 was not accompanied by the appearance of corresponding encapsidated ssRNA. The recent sudden appearance of encapsidated RNA 3 in the Wisconsin strains of PEMV suggests that RNA 3 may in fact have a significant involvement in PEMV infection. Thus, although RNA 3 does not appear to be essential for infectivity, it is not clear whether or not it participates in the infection process (subgenomic messenger), is an extraction artefact, a non-essential viral RNA or whether it is a satellite or defective interfering (DI) RNA.
To understand the genomic strategy of this unique virus, it became imperative to define specifically what genetic elements actually compose the full complement of the PEMV genome.

As discussed in recent review articles by Murant & Mayo (1982) and by Francki (1985), there are a number of extraneous genetic elements associated with plant RNA virus infections. These include subgenomic messengers, DI RNAs, satellite elements, viroids, virusoids and encapsidated host RNAs (pseudovirions). These elements can be distinguished by determining their nucleotide sequence relationship to viral and host genomes, and by assessing their replicative autonomy. We report here the use of a cloned cDNA probe specific for RNA 3 to examine these possibilities and conclude that RNA 3 is in fact a satellite RNA.

METHODS

Virus isolates. The aphid-transmissible Wisconsin NMT strain containing RNA 3 (hereafter designated PEMV+3) and the non-aphid-transmissible WSG strain containing RNA 3 (designated WSG+3) were propagated on *Pisum sativum* cv. 8221 by serial mechanical inoculation to 10-day-old seedlings at 3 to 4 week intervals. An RNA 3-deficient strain of PEMV NMT (designated PEMV-3) was generated through RNA inoculations using a combination of fractionated RNA 1 and 2 (see below) and maintained in cultivar 8221 in an isolated growth chamber under an identical serial inoculation scheme. A strain containing the satellite of cucumber mosaic virus (CMV+CARN A 5) was provided by C. J. Gabriel and propagated on *Nicotiana tabacum* cv. Turkish.

Virus purification. Virions of PEMV were purified by two cycles of differential centrifugation followed by sucrose gradient centrifugation as described previously (German & de Zoeten, 1975). Yields were consistently 10 to 15 mg virus/kg infected tissue.

Purification of viral RNA. Viral RNA was prepared using a modification of the protocol described by Gabriel & de Zoeten (1984). Bentonite (0.5 mg/mg virus), 20% SDS to a final concentration of 0.5% and proteinase K (Bethesda Research Laboratories) to a concentration of 100 μg/ml were added to the final resuspended virus preparation. The mixture was incubated at 37 °C for 20 min. SDS (20%) was added to a final concentration of 1% and the mixture was extracted repeatedly with Tris-saturated phenol:chloroform:isoamyl alcohol (24:24:1 ratio) until the interface was free of denatured protein. Five m-sodium acetate (pH 5.2) was added to a final concentration of 0.25 M, and the RNA was precipitated with 2.5 volumes of absolute ethanol at −80 °C.

RNA infectivity analysis. Individual PEMV RNAs were fractionated on 1.2% low melting point (LMP) agarose gels (Bethesda Research Laboratories) in 0.089 m-Tris, 0.089 m-boric acid, 0.002 m-EDTA running buffer (1 × TBE) as described by Maniatis et al. (1982). RNA bands were detected by staining in 0.5 μg/ml ethidium bromide and excised. Nucleic acids were recovered by remelting the agarose slices at 65 °C in an equal volume of a solution containing 400 mM-NaCl, 50 mM-Tris- HCl pH 7.5; 2 mM-EDTA followed by three successive extractions with 0.13 volume Tris-saturated phenol. The supernatant was desalted with three n-butanol washes before precipitation of the nucleic acids with ethanol.

Fractionated viral RNAs were resuspended in various combinations to approx. 0.2 mg/ml (0.1 ml volumes) in 10 mM-Tris- HCl pH 7.5, 1 mM-EDTA buffer supplemented at 1 mg/ml with EDTA-washed bentonite, and inoculated to 10-day-old pea seedlings dusted with carborundum (600 mesh).

Preparation of dsRNA of PEMV. Double-stranded RNAs from PEMV-infected, uninfected and mock-inoculated pea seedlings and of CMV-infected *N. tabacum* cv. Turkish were analysed by the method described by Morris et al. (1983) at 7 days post-infection. Double-stranded RNAs were purified through two cycles of CF-11 (Whatman) chromatography as described by Franklin (1966).

Electrophoresis of nucleic acids. Total cellular RNA, DNA and encapsidated viral RNA were analysed on 0.8 to 1.2% native agarose gels in 1 × TBE running buffer as described by Maniatis et al. (1982). Double-stranded RNA was separated by electrophoresis on 2.4% acrylamide-0.45% agarose composite gels as described by Peacock & Dingman (1968).

Cloning of PEMV RNA 3. Prior to the emergence of a single-stranded encapsidated form of RNA 3, a cDNA clone of RNA 3 was prepared using the dsRNA RF as template using a protocol similar to that developed by Revel et al. (1986) for cloning bacteriophage φ6 dsRNA. Double-stranded RNA was prepared and the RF of RNA 3 was isolated electrophoretically on a 1.2% LMP gel as described above, yielding approximately 8 μg RF. The RF of RNA 3 was polyadenylated in the ds state with 2 units of *Escherichia coli* poly(A) polymerase (Bethesda Research Laboratories) in 50 μl of a buffer consisting of 50 mM-Tris- HCl pH 7.9, 10 mM-MgCl2, 250 mM-NaCl, 0.25 mM-ATP, 500 μg/ml bovine serum albumin and 5 mM-dithiothreitol (DTT) at 37 °C for 30 min. Incorporation of [α-32P]ATP (400 Ci/mmol) was monitored in the reaction mixture (35 to 50 A residues added per 3' end). The polyadenylated product was phenol-extracted, ethanol-precipitated and resuspended in 10 μl water. One μl of 100 mM-methylmercuric hydroxide was added and the RNA was denatured at room temperature for 10 min.
Two μl of 700 mM-2-mercaptoethanol and 35 units RNase inhibitor (Promega Biotech) were added and the mixture was incubated at room temperature for 5 min to complex mercuric ions. The denatured polyadenylated RNA was made up to 50 μl in reverse transcriptase reaction buffer with a final composition of 50 μg/ml actinomycin D, 50 mM-Tris·HCl pH 8.3, 75 mM-KCl, 3 mM-MgCl₂, 0.5 mM each of dATP, dTTP, dGTP, 0.35 mM-dCTP, 2.5 μM-[α-32P]dCTP and 200 μg/ml oligo(dT)₁₂₋₁₈ (Pharmacia). Five μl of 20 units/μl cloned Moloney murine leukaemia virus reverse transcriptase (Bethesda Research Laboratories) was added and the mixture was incubated at 37°C for 60 min. The reaction was terminated by adding 2 μl 0.5 M-EDTA and the RNA template was hydrolysed by adding 25 μl of 150 mM-NaOH (65°C for 1 h). The mixture was neutralized with 25 μl of 1 M-Tris·HCl pH 8.0 and 25 μl of 1 M-HCl, and deproteinized by phenol extraction. The cDNA product was separated from residual non-reacted dNTPs by chromatography on Sephadex G-50 columns. cDNA excluded cDNA fractions were combined and ethanol-precipitated, yielding 2-7 μg TCA-precipitable product. The plus and minus cDNA strands were then annealed at a concentration of 250 μg/ml in a buffer consisting of 20 mM-Tris·HCl pH 8.0, 100 mM-NaCl, 1 mM-EDTA and 50% deionized formamide by heating to 80°C for 2 min and slow cooling to room temperature overnight (Cashdollar et al., 1982). The product was ethanol-precipitated and washed three times with 70% ethanol. The partially ds cDNA was completed with reverse transcriptase repair synthesis using the reaction conditions described above (in the absence of the oligo(dT) primer) followed by phenol extraction and ethanol precipitation. The cDNA product was size-fractionated on a 10% agarose gel and the primary cDNA product (approx. 750 bp) was located by autoradiography, excised, electroeluted and ethanol-precipitated. The cDNA was then dC-tailed (n = 20 to 25 dC residues) with terminal transferase (Boehringer) and annealed to dG-tailed puC19 at the PstI site (Vieira & Messing, 1982) as described by Maniatis et al. (1982). The annealed DNA was transformed into E. coli DH5α cells and recombinants were selected on the basis of a white colony and ampicillin resistance. ‘Minipreparation’ (alkaline lysis) analysis yielded three colonies with inserts of approx. 750 bp (hereafter designated pRNA3). These were increased, purified through CsCl density gradient centrifugation, and utilized as templates in nick translations (Maniatis et al., 1982) for Northern and Southern blot analyses.

**Analysis of total cellular DNA.** Total cellular DNA was isolated using an adaptation of the methods described by Dellaporta et al. (1983) and Murray & Thompson (1980). One g of healthy pea tissue (cvs. Perfected Wales and 8221) was ground to a fine powder in liquid nitrogen and suspended in 2 ml of 30 mM-EDTA, 1 M-NaCl, 1% hexadecltrimethyl ammonium bromide and 20 μg/ml proteinase K at 60°C for 30 min. The mixture was deproteinized with phenol:chloroform, adjusted to 2 M with 10 M-ammonium acetate and precipitated with 1 volume isopropanol. The fibrous mass was washed in 0.3 M-ammonium acetate-50% isopropanol followed by incubation in 10 mg/ml RNase A, 0.1% Sarkosyl, 10 mM-Tris·HCl pH 8.0, 10 mM-NaCl, 1 mM-EDTA for 15 min at 60°C. The mixture was extracted with phenol:chloroform and precipitated with isopropanol as stated above. Twenty μg of DNA was digested with 20 units HindIII and electrophoresed on a 0.8% agarose gel in 1 x TBE followed by electroblotting onto GeneScreen Plus membranes (New England Nuclear) following the manufacturer’s instructions. A control, consisting of 1-5 pg of the RNA 3-specific insert derived from PstI digestion of pRNA3 (the equivalent of one copy per pea genome; Murray et al., 1978) was included as a positive control.

**Total cellular RNA analysis.** Total plant cellular RNA was prepared using a modification of the procedure of Silliflow et al. (1979). One g of tissue (10 days post-infection) was ground in liquid nitrogen and vortexed in 5 ml of 50 mM-Tris·HCl pH 8.0 containing 4% para-aminosalicylic acid, 1% tri-isopropylphenylphosphonic acid, 10 mM-DTT and 10 mM-sodium bisulphite. The mixture was then deproteinized with Tris-saturated phenol followed by a second extraction with chloroform. LiCl was added to 3 M and the nucleic acids were precipitated overnight at −20°C. The resultant pellets were washed with 2 M-LiCl, and the RNAs were resuspended in water and ethanol-precipitated. Twenty μg aliquots were analysed by Northern blot analysis on analytical 1.2% agarose gels as described above.

**Aphid transmission studies.** Aphid transmission of PEMV isolates utilized the green peach aphid *Myzus persicae* (Sulzer) which had been reared on *P. sativum* L. cv. 8221. Three groups of 10 adult aphids were given 24 h acquisition feedings on infected pea plants 14 days after mechanical inoculation with either PEMV+3, PEMV−3 or WSG+3 strains. Following the acquisition feeding, single aphids were transferred to individual 7-day-old pea seedlings for a 24 h transmission feeding period. Transmission of PEMV was assessed at 14 days post-inoculation.

**Electron microscopy.** EM analysis of partially purified virion preparations was performed as described by Christie et al. (1987). Virions of PEMV+3 and PEMV−3 were purified as described above (omitting the sucrose gradient centrifugation), diluted to approximately 50 μg/ml in 0.1 M-sodium acetate pH 6.0 buffer, and applied to carbon-coated butvar membranes mounted on copper EM grids. Excess buffer was removed by wicking with a small corner of blotting paper, and the grid surface was sequentially washed dropwise with 1 ml of acetate dilution buffer followed by 1 ml of 250 μg/ml bacitracin (Sigma) in water. The grids were then negatively stained in an aqueous solution of 2% uranyl acetate containing 250 μg/ml bacitracin. The excess stain was removed with blotting paper, and the grids were air-dried. Electron microscopy was performed on a Hitachi H600 electron microscope.
RESULTS

Inter-relationships of encapsidated RNAs to dsRNA 3

In light of the asynchronous appearance of encapsidated RNA 3 and its putative RF, it was important to compare the nucleotide sequences of these two species. Fig. 1, lane 3 illustrates dsRNA analysis of PEMV + 3-infected pea tissue and the corresponding Northern blot probed with nick-translated pRNA3. This blot establishes the specificity of the probe to its original template. Fig. 2, lane 6 displays a similar Northern blot analysis of the encapsidated ssRNA generated from PEMV + 3 virions. The homology between the ds and ssRNA coupled with the size correlation argues strongly that the dsRNA is indeed an RF of the encapsidated ssRNA. As these figures also show, pRNA3 has no sequence homology to RNAs 1 and 2 or to their RFs. The lack of homology to PEMV genomic RNAs shows that RNA 3 is not a subgenomic messenger, a DI RNA or a PEMV genomic RNA fragment.

The non-essential role of RNA 3

By inoculating plants with electrophoretically fractionated RNAs, we have generated a strain of NMT PEMV devoid of RNA 3 (PEMV - 3). Lane 5 of Fig. 2 displays ssRNA isolated from PEMV - 3 following 12 successive passages in pea cultivar 8221. Lane 6 shows encapsidated RNA isolated from PEMV + 3 virions. Clearly, there is no evidence of encapsidated RNA 3 in Northern blot analysis at the levels supported by the RNA + 3 strain. Since we have described evidence that RNA 3 may be present but not detectably encapsidated, we have examined dsRNA and total cellular RNA from PEMV + 3 and PEMV - 3 infection. Fig. 2, lanes 3 and 4 display Northern analysis of total cellular RNAs isolated from PEMV - 3- and PEMV + 3-infected plants, respectively. Fig. 1, lanes 3 and 4 show dsRNA isolated from the PEMV + 3 and PEMV - 3 strains, respectively. In both cases, PEMV - 3 is devoid of RNA 3, confirming that RNA 3 is non-essential for PEMV infection (Gonsalves & Shepherd, 1972; Hull & Lane, 1973).

Lack of autonomous infectivity of RNA 3

The ability of RNA 3 to infect was assessed by direct RNA inoculation of identical aliquots of electrophoretically purified RNA 3 to healthy and PEMV - 3-infected pea seedlings. Lane 2 of
Fig. 2. (a) Agarose gel electrophoretic separation (1.2% agarose) of total cellular RNA (lanes 1 to 4) and encapsidated viral RNAs (lane 5 and 6). Lane 1, 20 µg total RNA isolated from mock-inoculated pea seedlings (*P. sativum* L. cv. 8221); lane 2, 20 µg total RNA isolated from uninoculated pea seedlings; lane 3, 20 µg total RNA isolated from PEMV-3-infected pea seedlings; lane 4, 20 µg total RNA isolated from PEMV+3-infected pea seedlings; lane 5, 5 µg encapsidated viral RNA isolated from purified PEMV-3 virions; lane 6, 5 µg encapsidated viral RNA isolated from purified PEMV+3 particles. (b) Northern blot analysis of same gel using nick-translated pRNA3 as probe.

Fig. 3 shows that RNA 3 inoculated to uninfected pea seedlings failed to exhibit the autonomous replication anticipated for a viroid element. Seedlings infected with PEMV-3 and superinfected with RNA 3 supported replication of RNA 3 at detectable levels in the first generation (Fig. 3, lane 1). These data also confirm the earlier results of Gonsalves & Shepherd (1972) and of Hull & Lane (1973), establishing that RNA 3 does not replicate independently.

**Examination of homology to host nucleic acids**

Fig. 2, lanes 1 and 2 show Northern blot analysis of total cellular RNAs generated from mock-inoculated and healthy 8221 seedlings. Fig. 1, lanes 5 and 6 display a similar result from Northern blot analysis of dsRNA generated from healthy and mock-inoculated pea seedlings. There is no homology between host RNAs and RNA 3, suggesting that RNA 3 has not arisen from the encapsidation of a host RNA (pseudovirion) or from an inoculation- or stress-induced RNA. To confirm that RNA 3 is not homologous to a low copy number host RNA, we probed total plant DNA generated from cultivars 8221 and Perfected Wales (which had been used previously to maintain the Wisconsin PEMV strains). As is evident in Fig. 4, lanes 2 and 3, there
Fig. 3. (a) Agarose gel electrophoretic separation (1.2% agarose) of total cellular RNA (lanes 1 and 2) and virion encapsidated RNA (lane 3 and 4). The positions of size markers ($M_r \times 10^{-6}$) consisting of PEMV and BMV genomic RNAs are shown at the left-hand side of the gel. Lane 1, 20 µg total cellular RNA isolated from PEMV-3-infected pea seedlings superinfected with RNA 3; lane 2, 20 µg total cellular RNA isolated from pea seedlings inoculated with RNA 3 alone; lane 3, 5 µg encapsidated RNA isolated from purified PEMV-3 virions; lane 4, 5 µg encapsidated RNA isolated from PEMV+3 virions; lane 5, 5 µg brome mosaic virus RNA marker. (b) Northern blot analysis of corresponding gel probed with nick-translated pRNA3.

is no detectable homology to healthy plant DNA at a level of sensitivity capable of detecting one copy of pRNA3 supplied artificially (lanes 4 and 5). Therefore, RNA 3 does not result from encapsidation of host genetic elements.

Examination of particle morphology

Fractionation of PEMV+3 virions purified by sucrose gradient centrifugation failed to demonstrate evidence of a unique particle not present in similar preparations of PEMV-3. Electron microscopy of virions derived from these two strains (before gradient fractionation) also failed to demonstrate a particle of unique size and morphology. All particles measured ($n = 100$) fell in the 25 to 30 nm diameter size range characteristic of PEMV particles. The lack of an RNA 3-encoded coat protein is also substantiated by preliminary sequence analysis of pRNA3 which shows that the largest open reading frame encodes 73 amino acids, an amount
PEMV satellite RNA

Fig. 4. (a) Agarose gel electrophoretic separation (0.8% agarose) of HindIII-digested total cellular DNA isolated from healthy pea tissue. The positions of HindIII-digested lambda size standards (Mr x 10^-6) are expressed at the left hand side of the gel. Lane 1, 1 µg HindIII-digested lambda DNA molecular weight marker; lane 2, 20 µg total cellular DNA isolated from P. sativum L. cv. 8221; lane 3, 20 µg total cellular DNA isolated from P. sativum L. cv. Perfected Wales; lane 4, 20 µg total cellular DNA from P. sativum L. cv. 8221 supplemented with 1.5 pg of the RNA 3-specific PstI insert of pRNA3 (the equivalent of one copy of RNA 3 encoding material per genome); lane 5, DNA marker consisting of 1-5 pg of the RNA 3-specific PstI insert of pRNA3 (identical to the quantity added in lane 4). (b) Corresponding Southern blot hybridization probed with nick-translated pRNA3. Exposure to X-ray film was for 14 days.

that is probably insufficient to encapsidate a unique particle. This would confirm the earlier report of Gonsalves & Shepherd (1972) who assigned the encapsidation of RNA 3 to a specific PEMV particle. These data, coupled with the inability to establish a definitive relationship between RNA 3 and the host and viral genomes, argue strongly that RNA 3 represents a satellite RNA of PEMV.

Phenotypic comparison of PEMV + 3 and PEMV − 3

No symptom differences were discernible between 8221 seedlings inoculated with PEMV + 3 or PEMV − 3 strains. This agrees with earlier studies (Gonsalves & Shepherd, 1972; Hull & Lane, 1973) which failed to establish a unique phenotype associated with this RNA. We also compared aphid transmissibility of these two strains in regard to the presence or absence of RNA 3. PEMV is transmitted both mechanically and by aphids in a circulative manner.
Repeated mechanical transmission of PEMV often eliminates vector transmissibility, which is accompanied by the loss of a distinctive component of the virion particle (Adam et al., 1979; Hull, 1977). Lemaire et al. (1988) showed a correlation between the loss of vector transmission of beet necrotic yellow vein virus and the deletion of the two smallest RNAs of the viral genome; we therefore examined whether a similar phenomenon may have been in effect with our PEMV isolates. Five out of 10 aphids transmitted PEMV + 3 and six out of 10 transmitted PEMV − 3 to healthy pea seedlings. In the case of the WSG + 3 strain, none of 10 aphids transmitted the WSG + 3 strain, consistent with its described non-transmissible nature. Northern blot analysis of total cellular RNA isolated from composite samples of aphid-inoculated plants confirmed that the RNA 3 status of the acquired strain was retained through aphid transmission (data not shown). Thus, RNA 3 has no detectable effect on aphid transmissibility of PEMV. Other effects of satellites, such as reduced viral replication, alterations in viral RNA proportions, and strain-host interactions (see Murant & Mayo, 1982; Francki, 1985) have not been examined for this satellite element.

DISCUSSION

The early infectivity studies of Gonsalves & Shepherd (1972) and Hull & Lane (1973) provided initial evidence of a small, non-essential RNA associated with the virions of PEMV-infected plants. Our infectivity analysis confirmed both the lack of infectivity and the non-essential nature of this RNA, and further demonstrated the total dependence of RNA 3 on the PEMV genome. We found, in addition, that RNA 3 has no homology to the host or helper genomes, ruling out the possibilities that RNA 3 is a subgenomic messenger, a DI RNA, a viral RNA artefact or a pseudovirion. Coupled with the lack of evidence for a distinct RNA 3-associated virion and lack of an RNA 3-encoded phenotype, the data unequivocally establish that PEMV RNA 3 is a satellite RNA. We can therefore add the monotypic PEMV virus group to the rapidly expanding list of satellite-supporting virus groups.

The presence of a satellite RNA associated with PEMV represents some interesting avenues for further research. PEMV is one of the few plant RNA viruses for which a replication complex has been defined, in this case originating in vesicles derived from the nuclear membrane (de Zoeten et al., 1972). This complex can be examined by electron microscopy in situ labelling techniques and by precursor incorporation in vitro (de Zoeten et al., 1976; Powell & de Zoeten, 1977; Powell et al., 1977). If, as is hypothesized, satellites interfere with RNA replication (Kaper, 1982; Piazzolla et al., 1982), then, they are a prime tool for studying RNA replication. The accessibility of an active viral replicative fraction should provide an enzymatic and cytological means for examining such competition and would provide an interesting contrast to the other satellite-helper combinations.

A second intriguing point is the delay between the appearance of RF and of an encapsidated ss satellite RNA. There are several explanations for this observation. One possibility is that the initial production of the encapsidated strand of RNA 3 was inefficient, and that a mutation at a specific locus led to the sudden increase seen in our strain. Since our cloned dsRNA was produced before this ssRNA increase, sequence comparisons of the current encapsidated satellite and the cloned probe should provide information on the domain(s) involved in such a mutational event. An alternative explanation would centre on a similar mutational event at a locus controlling encapsidation efficiency. It may be that the encapsidated satellite was in fact present at levels below the physical limits of detection, a phenomenon that has been described for a number of virus–host combinations (Garcia-Luque et al., 1984). Again, sequence comparisons of current and past satellites would shed light on the determinants of such an event. A third possibility also involving encapsidation is that in the early phase of satellite development, the satellite lacked determinants for encapsidation altogether. Since the stability of satellites is well documented (Mossop & Francki, 1979), and since this strain was propagated purely by serial mechanical inoculation, a replicatively active yet encapsidation inactive satellite precursor could have passed from generation to generation via mechanical inoculation much like viroids, until an active encapsidation signal evolved. This would imply that encapsidation of the satellite carried a selective advantage, such as the co-delivery of a full
genomic complement plus the satellite at the infection site. Such a role for mechanical inoculation may be important in the early phases of satellite evolution, and may shed light on the spontaneous development of satellites in isolation conditions (Francki, 1985). Again, sequence comparison between pRNA3 and the current active PEMV satellite should be instructive.

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