Available online at www.sciencedirect.com
SCIENCE DIRECT
Virology 345 (2006) 532 – 539
VIROLOGY
www.elsevier.com/locate/yviro

The genomic RNA packaging scheme of *Red clover necrotic mosaic virus*

Veronica R. Basnayake [1], Tim L. Sit, Steven A. Lommel *

Department of Plant Pathology, North Carolina State University, Raleigh, NC 27695, USA

Received 25 April 2005; returned to author for revision 19 May 2005; accepted 18 October 2005
Available online 17 November 2005

Abstract

Red clover necrotic mosaic virus (RCNMV) is a small icosahedral plant virus with a bipartite RNA genome. While the RCNMV genome consists of two RNAs, it has not been definitively established whether these RNAs are co-packaged into a single virion or packaged individually into separate virions. Biochemical evidence exists to support both hypotheses. To determine the genomic RNA complement within RCNMV, virions were subjected to heat treatments and UV crosslinking. A stable RNA-1:RNA-2 heterodimer was formed with both treatments establishing that RCNMV genomic RNAs are co-packaged into a single virion. Furthermore, RNA-2 homodimer and homotrimers were also observed indicating that some virions contain multiple copies of RNA-2 exclusively. These results indicate that RCNMV virions consist of two distinct populations: (i) virions containing both genomic RNAs; and (ii) virions with multiple copies of RNA-2. This type of hybrid packaging arrangement was unexpected and appears to be unique among the multipartite RNA viruses.

Keywords: RCNMV; Virions; UV crosslinking; Heating; Co-packaging; Genome; Multipartite; Heterodimer; Homodimer

Introduction

A key terminal step in the life cycle of viruses is the encapsidation of the viral genome by capsid protein (CP) subunits and the formation of mature virions. The genomes of RNA viruses can be either monopartite or multipartite. While monopartite genomes are obviously packaged into single virions, multipartite genomes face the additional problem of ensuring that all of the various genome segments are packaged into single virions or multiple, distinct virion species (van Regenmortel et al., 2000). For example, the Nodaviridae and Reoviridae encapsidate their multipartite genomes into single virions (Patton and Spencer, 2000; Qiao et al., 1997; Krishna and Schneemann, 1999; Selling and Rueckert, 1984) whereas the Bromoviridae utilize a more complex packaging mechanism to encapsidate the three genomic RNAs and a subgenomic RNA (sgRNA) into three separate virions of identical size and morphology (Choi and Rao, 2003).

Red clover necrotic mosaic virus (RCNMV) is a small spherical RNA plant virus in the *Dianthovirus* genus, family *Tombusviridae*. RCNMV virions are non-enveloped and contain 180 copies of the 37 kDa CP subunit to form an isometric particle of $T = 3$ symmetry, 36 nm in diameter (Hamilton and Tremaine, 1996; Xiong and Lommel, 1989). The RCNMV genome consists of two single-stranded positive-sense RNAs, RNA-1 and RNA-2 (Fig. 1A). The 3.9 kb RNA-1 codes for three proteins: (i) p27, a polymerase related protein, (ii) p88, the RNA-dependent RNA polymerase, and (iii) the CP (Xiong and Lommel, 1989; Xiong et al., 1993b). The polymerase proteins are translated directly from RNA-1 while the CP is expressed from a sgRNA (Zavriev et al., 1996). The 1.5 kb RNA-2 is monocistronic and codes for the movement protein required for cell-to-cell movement (MP; Lommel et al., 1988; Osman and Buck, 1991; Xiong et al., 1993a). RNA-2 is also required for the initiation of CP sgRNA synthesis from RNA-1 (Sit et al., 1998). A 34-nucleotide stem loop structure, termed the *trans*-activator (TA), on RNA-2 base pairs with RNA-1 at an 8-nucleotide element termed the TA binding site (TABS) within the CP subgenomic promoter. This low affinity binding event directs the synthesis of the sgRNA presumably via a premature termination mechanism during minus strand synthesis of full-length RNA-1 (Guenther et al., 2004; Sit et al., 1998; White, 2002).

* Corresponding author. Fax: +1 919 515 7169.
E-mail address: steve_lommel@ncsu.edu (S.A. Lommel).
[1] Present address: School of Biological Sciences, University of Nebraska-Lincoln, E229 Beadle Center, Lincoln, NE 68588-0666, USA.

0042-6822/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2005.10.017

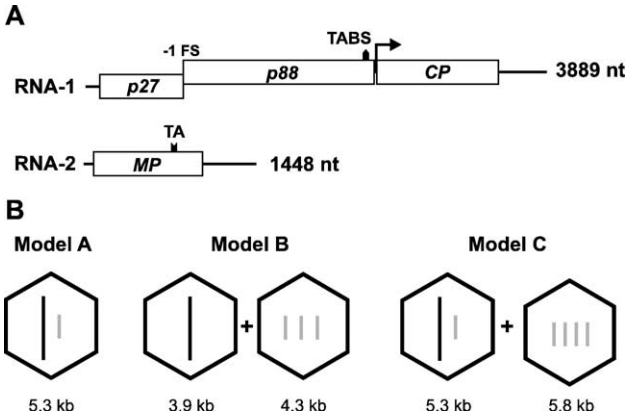

Fig. 1. RCNMV genome organization and hypothetical packaging schemes for the genomic RNAs. (A) Schematic representation of the RCNMV genome. Open reading frames are depicted as boxes with the encoded products indicated within. p27 and p88 are the polymerase-associated proteins, CP is the capsid protein, and MP the movement protein. The −1 ribosomal frameshift signal (−1 FS) that produces p88 from p27 is indicated. (B) Various schemes for the packaging of RCNMV genomic RNAs into virions. Model A displays only one virion type containing a copy of each RNA together in a single virion. Model B displays two virion types: one with a single copy of RNA-1 and the other with 3 copies of RNA-2. Model C, a hybrid of Models A and B, displays two virion types: one with a copy of each of RNA-1 and RNA-2 and the other with 4 copies of RNA-2.

The genomic RNA complement of RCNMV virions has yet to be determined unequivocally. Purified virions form a single band in CsCl density gradients indicating that the entire virion population is of uniform or nearly uniform density (Gould et al., 1981; Hollings and Stone, 1977). However, RNA purified from RCNMV virions or the type species of the *Dianthovirus* genus, *Carnation ringspot virus* (CRSV) exhibit an RNA-1:RNA-2 ratio of approximately 1:3 as determined by sucrose gradient fractionation studies, suggesting that more RNA-2 is packaged than RNA-1 (Hamilton and Tremaine, 1996; S. A. Lommel, unpublished data). Finally, an infectivity-dilution assay of CRSV yielded two hit kinetics, suggesting that the biologically active virus is composed of two virion species (Hamilton and Tremaine, 1996). Based on these incongruent observations, there are currently two competing hypotheses for the packaging of the RCNMV genome (Fig. 1B). The first (Fig. 1B, Model A) is based on the equilibrium density gradient findings that suggest the co-packaging of both genomic RNA-1 and -2 into a single virion (Gould et al., 1981; Hollings and Stone, 1977). This type of virion would have an RNA content of approximately 5.3 kb. The second hypothesis (Fig. 1B, Model B) is based on analysis of the RNA content of virions and the infectivity-dilution data which suggest the presence of two distinct virions, each packaging either genomic RNA. In this model, it is proposed that one type of virion contains a single copy of RNA-1 (3889 nt) while the other type of virion would contain three copies of RNA-2 (4344 nt). The disparity of 455 nt in RNA content, and thus virion density, between the two types of virions would not likely be distinguishable in CsCl density gradients. This assumption is supported by the fact that *Brome mosaic virus* (BMV) yields a single band of 1.35 g/cm^3 in CsCl equilibrium centrifugation yet is composed of three virion species packaging 3234, 2865, and 2993 nt of RNA, for a difference of up to 369 nt between virions (Ahlquist et al., 1984; Lane, 1977).

Intriguingly, a hybrid model combining elements of both hypotheses can be envisioned (Fig. 1B, Model C). One type of virion would contain both genomic RNAs (5337 nt) comprising a biologically active virion, while the other type would package four RNA-2 molecules, giving rise to virions with an RNA content of 5792 nt. Given the size of the RCNMV virion and the resulting physical packaging constraints, it is unlikely that it could package more than 5800 nt of RNA.

To determine the true RCNMV RNA complement, virions were subjected to both heat treatment and UV irradiation. Heat treatment of *Flock house virus* (FHV; *Nodaviridae*) particles resulted in the formation of an RNA complex between the two genomic RNAs showing that they are co-packaged (Krishna and Schneemann, 1999). UV irradiation crosslinks contacting and closely aligned RNAs and has been successfully used to determine the RNA complement within icosahedral virions (Mayo et al., 1973; Newman and Brown, 1977). After either treatment, RCNMV RNA-1:RNA-2 heterodimers as well as RNA-2 homodimers and multimers were formed. These results are only possible if the two dimerized RNAs are in close proximity. Given the assumption that RCNMV cannot stably package greater than 5800 nt of RNA, we conclude that a proportion of the RCNMV virions co-package RNA-1 and RNA-2 into a single virion while another fraction of the virion population packages 4 RNA-2s exclusively. This conclusion supports the hybrid model (Fig. 1B, Model C) which has not been previously observed for any other icosahedral RNA virus with a multipartite genome.

Results

High molecular weight RNA species are formed upon heating of RCNMV virions

Krishna and Schneemann (1999) employed a novel heating approach to determine the RNA content within FHV virions. The bipartite RNA genome of FHV is encapsidated within an icosahedral virion similar in size to RCNMV. Following the protocol of Krishna and Schneemann (1999), RCNMV virions were incubated over a range of temperatures from 35–95 °C. Virion RNAs were extracted and subjected to semi-denaturing electrophoresis. RNA species larger than either genomic RNA were observed from virions treated at temperatures between 45 and 85 °C but not in the unheated control (Fig. 2A). Optimal accumulation of these high molecular weight RNA species was observed at 65 °C with decreasing efficiencies of formation above and below this temperature. These RNA species appear to be larger than 5 kb in size (Fig. 2A). The heat treatment did not result in any obvious morphological changes to the RCNMV virions (Fig. 2B) suggesting that the higher molecular weight

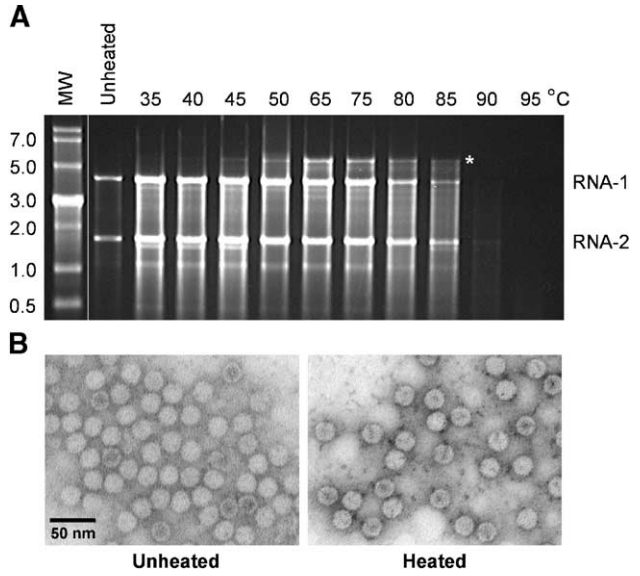

Fig. 2. A high molecular weight RNA species is formed upon heating of RCNMV virions. (A) Temperature range for high molecular RNA species formation. RCNMV virions were heated at the noted temperatures prior to virion RNA extraction and gel electrophoresis on a 1% TBE agarose gel. The location of the higher molecular weight band is denoted with an asterisk. RNA sizes (in kb) are indicated to the left. (B) Electron micrographs of negatively stained RCNMV virions. RCNMV virions were either untreated or heated at 65 °C for 10 min prior to application on copper grids, followed by staining with 2% uranyl acetate and observation.

RNA species is formed within the virions as a result of the heating process.

RCNMV RNA-1 and RNA-2 are present in the high molecular weight RNA species formed upon heat treatment of virions

To verify the composition of this high molecular weight RNA species, Northern blot hybridization analysis was performed on viral RNA extracted from RCNMV virions heat treated at 65 °C. Duplicate blots were hybridized separately with RNA-1 and RNA-2 specific probes. As expected, no larger RNA species are detectable in the unheated controls. The larger RNA species generated upon virion heating does hybridize to both probes indicating that it consists of both RCNMV genomic RNAs (Figs. 3A and B, middle and lower panels). The size of this RNA species is approximately 5.3 kb which corresponds to the combined sizes of RNA-1 and RNA-2. This suggests the formation of a heterodimer complex between the two genomic RNAs of RCNMV when virions are heated at 65 °C as was observed for FHV. This heterodimerization can only occur when the RNAs are in close proximity, indicating that a portion of the RCNMV virion population appears to co-package RNA-1 and RNA-2 into the same virion.

Two duplicate experiments are presented in Figs. 3A and B to illustrate two different points. 80% of the time, essentially all of RNA-1 and a large percentage of RNA-2 dimerized and shifted into the higher band as seen in Fig. 3B. Coincident with this high level of heterodimerization within the virion is the difficulty in resolving intermediate multimeric forms. Periodically, in 20% of the heat treatment experiments, high resolution of intermediate multimers is achieved as seen in Fig. 3A which correlated with a lower percentage of the RNAs forming the heterodimer.

Northern blot analysis revealed the presence of additional intermediate RNA species consisting solely of RNA-2 (Fig. 3A, lower panel). These RNA species are approximately 3 and 4.5 kb in size corresponding to 2 and 3 copies of RNA-2, respectively. Thus, heating of RCNMV virions generates not only a predominant RNA-1:RNA-2 heterodimer but also, unexpectedly, RNA-2 multimers. An additional band was observed on the blot probed with RNA-1 (Fig. 3A, middle panel) that corresponds to the size of RNA-2. This band was not observed in any other blots probed with RNA-1 and is presumed to be an artifact.

RNA multimers are formed within RCNMV virions

Despite the lack of obvious sequence complementarity between the RCNMV genomic RNAs, an RNA-1:RNA-2 heterodimer complex was formed. RCNMV RNA transcripts as well as purified virion RNA were annealed at 65 °C in vitro in the absence of CP to examine the possibility of genomic RNA complex formation outside of the virion. No RNA complexes were observed with any combination of genomic RCNMV transcripts (Fig. 4). Similarly, no RNA complexes were

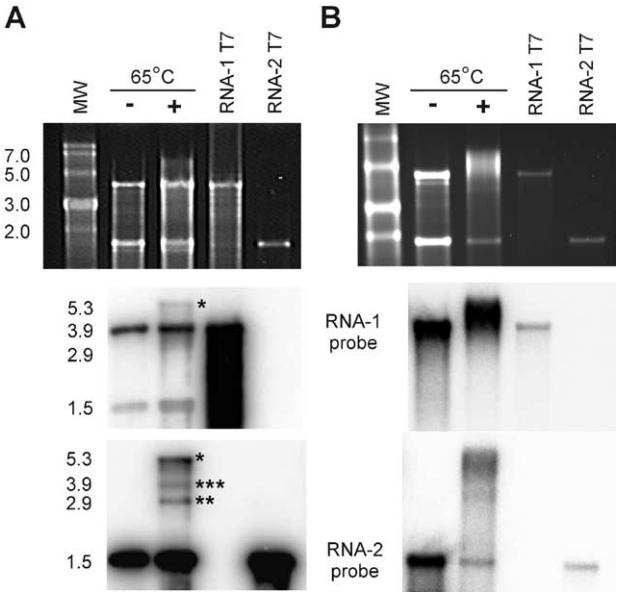

Fig. 3. RCNMV virions heated at 65 °C produce RNA-1:RNA-2 heterodimers and RNA-2 multimers. Virion RNAs from unheated and heated samples were phenol extracted, electrophoresed through a non-denaturing 1% TAE agarose gel (top panels), and analyzed by Northern hybridization. Probes specific to RCNMV RNA-1 (middle panels) and RNA-2 (bottom panels) were used for hybridizations. Full-length T7 RNA transcripts representing genomic RNAs-1 and -2 served as controls. A single asterisk identifies the RNA-1:RNA-2 heterodimer band. A double asterisk identifies the RNA-2 homodimer and the triple asterisk denotes the RNA-2 homotrimer bands. The RNA sizes (in kb) are indicated to the left. (A) Depicts a minority profile from 2 of 10 repetitions of the experiment where RNA-1:RNA-2 heterodimer formation was limited and RNA-2 homodimers and multimers were resolved. (B) A typical dimerization profile from 8 of 10 repetitions where essentially all of RNA-1 and most of RNA-2 heterodimerized.

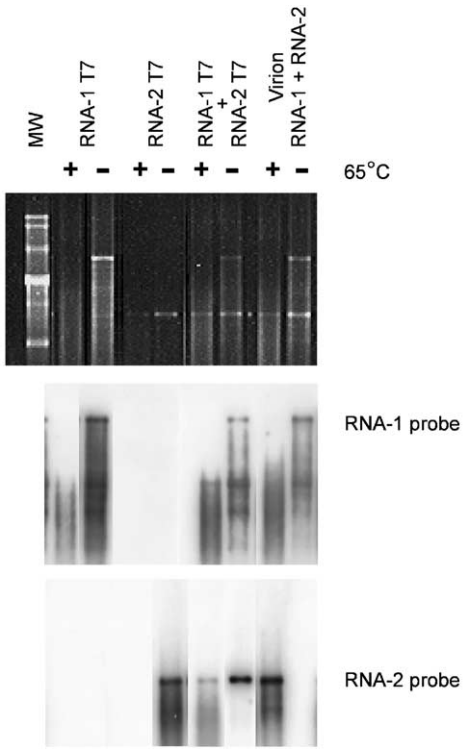

Fig. 4. Annealing of RCNMV transcripts or purified viral RNA does not result in the formation of high molecular weight RNA species. RCNMV RNA-1 and RNA-2 transcripts as well as purified virion RNAs were heated to 65 °C for 10 min followed by immediate cooling. The top panel is a 1% TAE agarose gel after electrophoresis of heated and unheated RNAs. Middle panel is a Northern blot of the gel hybridized with an RCNMV RNA-1 probe and the bottom panel is a Northern blot of the gel hybridized with an RCNMV RNA-2 probe. As seen on the Northern blots, no RNA species larger than RNA-1 was detected.

detected upon annealing of purified virion RNA (Fig. 4). These results suggest that RNA complexes are only formed in the presence of CP or, more likely, only within intact virions.

RNA complex formation by UV irradiation of RCNMV virions reinforces earlier findings from virion heat treatments

Irradiation of RNAs by far UV light results in crosslinking only when RNAs are in close proximity through either transient contacts or base pairing interactions (Juzumiene et al., 2001; Whirl-Carrillo et al., 2002). These RNA crosslinks are formed by covalent interactions and are stable under denaturing conditions. UV irradiation of RCNMV virions resulted in the formation of a ~5.3 kb heterodimer complex containing RNA-1 and RNA-2 (Fig. 5) as was observed with the heat treatment of virions. This strengthens our conclusion that RNA-1 and RNA-2 are co-packaged into a single virion.

Additional bands corresponding to multimers of RNA-2 (~3.0 and 4 kb) were also observed (Fig. 5). The formation of RNA-2 multimers by UV treatment firmly establishes the packaging of multiple copies of RNA-2 within a single virion. Formation of heterodimers and RNA-2 multimers by UV crosslinking of whole virions reinforces the findings obtained by heat treatment of virions.

The RNA-1:RNA-2 heterodimer complex is formed by a non-covalent interaction

The RCNMV heterodimer complexes may be produced by either covalent or non-covalent RNA interactions. The definitive nature of this interaction can be established by subjecting these RNA complexes to fully denaturing gel electrophoresis which would disrupt any non-covalent interactions. The heterodimer complex formed during heat treatment did not persist under denaturing conditions (Fig. 6) revealing that this RNA complex was maintained by non-covalent interactions. As expected, the UV induced heterodimer complex was not disrupted by fully denaturing gel electrophoresis due to the covalent nature of the UV crosslinks. This unequivocally establishes the non-covalent nature of the heterodimer complex formed by virion heating.

Discussion

Heat and UV treatment of intact virions has been successfully used to determine the RNA content packaged within icosahedral virions. Heat treatment of FHV virions and

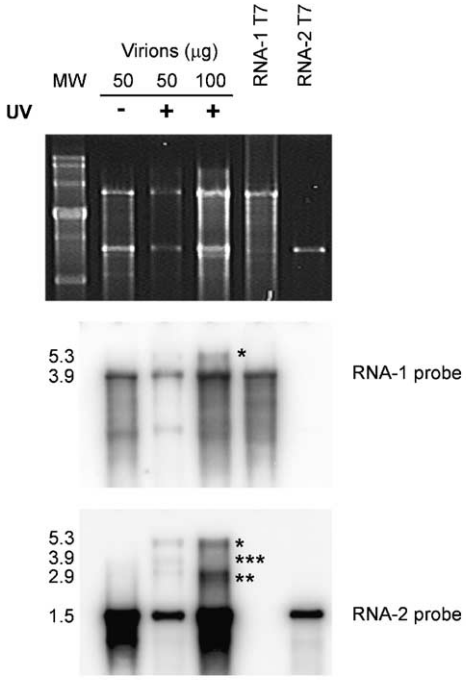

Fig. 5. UV irradiation of RCNMV virions results in the formation of RNA-1:RNA-2 heterodimers and RNA-2 multimers. Top panel; RCNMV virions (50 µg and 100 µg aliquots) were UV-crosslinked for 20 min prior to virion RNA extraction and electrophoresis in a 1% agarose TAE gel. Northern blots were probed with genomic RNA specific probes. The middle panel is a Northern blot hybridized with an RCNMV RNA-1 probe and the bottom panel a Northern blot hybridized with an RCNMV RNA-2 probe. Various RNA multimers are denoted by asterisks: single asterisk identifies the RNA-1:RNA-2 heterodimer band, the double asterisk the RNA-2 homodimer band, and the triple asterisk the RNA-2 homotrimer. RNA from untreated virions and T7 RNA transcripts of the respective genomic RNAs served as controls. RNA sizes (in kb) are indicated to the left.

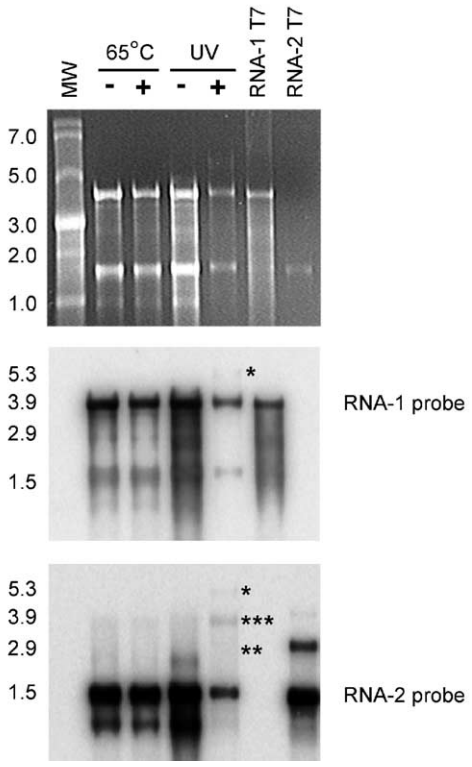

Fig. 6. UV-crosslinked RCNMV virion RNA but not heat treated virion RNA remains complexed after denaturation. Virion RNA extracted from both heat treated and UV-crosslinked RCNMV virions were electrophoresed in a denaturing 1% formaldehyde agarose gel (top panel) and analyzed by Northern blot hybridization with RCNMV RNA-1 (middle panel) and RNA-2 (bottom panel) probes. A single asterisk identifies the RNA-1:RNA-2 heterodimer band. The double asterisk identifies the RNA-2 homodimer and the triple asterisks the RNA-2 homotrimer bands. T7 RNA transcripts were included as controls. RNA sizes (in kb) are indicated to the left.

UV treatment of *Nodamura virus* and *Raspberry ringspot virus* were utilized to characterize the RNA complement within virions (Krishna and Schneemann, 1999; Mayo et al., 1973; Newman and Brown, 1977). Heating results in the rearrangement of the virion RNAs while UV treatment directly cross-links the RNAs leading to the production of genomic RNA dimers. That this occurs with either treatment confirms the co-packaged nature of the genomic RNAs within single virions. Heat and UV treatment of RCNMV virions leads to the formation of RNA-1:RNA-2 heterodimers as well as RNA-2 multimers without any apparent changes to virion morphology or integrity (Fig. 2B). Furthermore, the infectivity of heat treated RCNMV virions did not appear to be severely reduced since these treated virions produced symptoms similar to an untreated wild-type control on *Nicotiana benthamiana* (data not shown).

The formation of RNA heterodimers and homodimers within virions is not a phenomenon unique to Nodaviruses and RCNMV. Genomic homodimers occur naturally and are a requirement for *Human immunodeficiency virus* type 1 (HIV-1) encapsidation (Laughrea et al., 1997; Sakuragi et al., 2003). HIV-1 RNA dimers are preferentially packaged while monomeric RNAs are excluded (Fu and Rein, 1993; Fu et al., 1994). HIV-1 dimerization occurs at the dimer initiation site (DIS), which is structurally linked to the encapsidation signal (ψ) to ensure packaging of dimeric HIV-1 (Fu et al., 1994; Sakuragi et al., 2003). Dimerization is required to expose ψ to be recognized by the nucleocapsid protein to initiate encapsidation (Sakuragi et al., 2003). In RCNMV, the TA element on RNA-2 initiates CP sgRNA synthesis from RNA-1 by base pairing to the TABS within the RNA-1 CP subgenomic promoter (Sit et al., 1998; Fig. 1A). Recently, it was demonstrated that the TA-TABS interaction forms a stacked helical structure (Guenther et al., 2004) which is structurally similar to the kissing hairpin loop structure of the HIV-1 DIS (Mujeeb et al., 1998). This may reflect a structure–function similarity between the HIV-1 kissing loop and the RCNMV TA-TABS structure that ensures heterodimer formation between RCNMV RNA-1 and RNA-2 for co-packaging.

Although the TA-TABS interaction is proposed to be the initial contact point for RCNMV RNA dimerization, it is not beyond the realm of possibility that additional points of interaction between RCNMV RNA-1 and RNA-2 exist. However, extensive stretches of complementarity between RNA-1 and RNA-2 do not exist. In the case of RNA-2 homodimers and multimers, an interaction similar to the TA-TABS or other less obvious sequences could be involved in RNA-2 homodimerization especially through non-Watson-Crick base pairing. CP may recognize a similar sequence/structure element common to both the RNA-1:RNA-2 heterodimer as well as the RNA-2 homodimer to initiate assembly. If RNA-2 homodimers are packaged, how does this explain the observed multimeric forms of RNA-2 and the expected packaging of four copies of RNA-2 per virion? It can be envisioned that the RNA-2 only virions are slightly less stable than RNA-1:RNA-2 heterodimer virions. There is precedence for this supposition based on SDS treatment of the dianthovirus, CRSV (Tremaine and Ronald, 1976). These studies illustrated the preferential liberation of RNA-2 from virions at low SDS concentrations prior to RNA-1 release at higher SDS concentrations. Thus, formation of RNA-2 homotetramers upon UV irradiation or heating may not have been favored whereas dimeric and trimeric forms of RNA-2 were more likely.

Based on non-denaturing gel electrophoresis, the RCNMV heterodimer complex is formed by a non-covalent interaction. Interestingly, in HIV-1, heat treatment melts the intra-strand base pairs comprising the DIS stem and induces the formation and extension of the homodimer (Laughrea and Jette, 1996; Muriaux et al., 1996b). Attempts to generate RCNMV RNA-1:RNA-2 heterodimers in vitro utilizing either RNA transcripts or purified viral RNA proved unsuccessful. However, we cannot rule out the possibility that the failure to generate dimers was due to RNA degradation upon heating/annealing as observed for RNA-1 transcripts (Fig. 4). HIV-1 transcripts have been shown to spontaneously dimerize in vitro (Darlix et al., 1990; Feng et al., 1996). HIV-1 nucleocapsid protein chaperones homodimer formation (Feng et al., 1996; Muriaux et al., 1996a). The RCNMV RNA annealing conditions employed in this study may require optimization through the addition of other components such as CP whose absence could be a limiting factor.

The formation of genomic RCNMV RNA dimers occurring within the confines of the virion clearly shows that at least two RNAs are co-packaged per RCNMV virion. Regardless of the treatment involved (either heating or UV irradiation), the same spectrum of RNA multimer species was generated. These include RNA-1:RNA-2 heterodimers and various RNA-2 multimers. From the multiple repetitions of the virion heating experiments depicted in Fig. 3, it is clear that most of the virions contain one copy each of RNA-1 and RNA-2 co-packaged and a minority of the virions contain multiple copies of RNA-2. We made numerous attempts to determine the accumulation ratio of the two virion populations by various forms of differential isopycnic and sedimentation ultra-centrifugation, without success (data not shown). Given this failure, we are unable to provide the relative percentage of each virion population that accumulates in infected cells. We can infer, however, by combining several lines of indirect evidence, the ratio of the two virion populations. To maintain the RNA-1:RNA-2 ratio of 1:3 liberated from purified virus, virions containing both genomic RNAs account for 67% of the population while RNA-2 only virions (containing 4 copies of RNA-2) make up the rest giving a virion ratio of 2:1, respectively. This calculation is consistent with the level of heterodimerization seen in Fig. 3B.

The results of this study have shown that the RCNMV genome is packaged into two distinct virion populations: (i) virions co-packaging RNA-1 and RNA-2 and (ii) virions containing multiple copies of RNA-2 as proposed in Model C (Fig. 1B). This supports the infectivity-dilution assays for dianthoviruses suggesting the presence of two distinct types of virions (Hamilton and Tremaine, 1996). Our findings both support and refute the earlier interpretations of CsCl density gradient data where RCNMV virions formed a single equilibrium band suggestive of an entire population of identical virions with equal density (Hollings and Stone, 1977). Virions obtained from this single band yielded a wild-type infection suggesting that either (i) both genomic RNAs were co-packaged into a single virion or (ii) virions contain differing genomic RNA complements but possess similar densities. Since RNA-2 only virions must have a similar density to virions encapsidating both RNA-1 and RNA-2 (5337 nt), it is likely that RNA-2 only virions contain four copies of RNA-2 (5792 nt) (Fig. 1B). This difference in total RNA content per virion (~8%; 455 nt) may not manifest itself in an observable difference in density on a CsCl gradient. This was the case for BMV where the difference in RNA content among the three different virions is ~11% (369 nt) yet they all band together in CsCl (Lane, 1977).

Our conclusions for the packaging scheme of RCNMV are based on the data provided here and on an assumption for the packaging capacity of the virion. Hamilton and Tremaine (1996) estimated the molecular mass of a single RCNMV virion to be 8.35×10^6 Da with the RNA contributing 20% of the mass (Hollings and Stone, 1977). These estimates, based on constituent mass calculations and UV spectra, correspond quite well with a virion containing one copy of each genomic RNA (8,322,954 Da) and 21% of the mass being the RNA or a virion containing four copies of RNA-2 (8,472,420 Da) and 22% RNA calculated from the complete nucleotide sequence of the RCNMV genome (Lommel et al., 1988; Xiong and Lommel, 1989). An RNA-1 only containing virion (7,851,084 Da) is only 16% RNA, which is too low and a virion with one copy of RNA-1 and two or more copies of RNA-2 (8,794,824 Da) would be 25% RNA or greater. The CP and RCNMV virion is phylogenetically and structurally conserved with *Tomato bushy stunt virus* (TBSV) and other tombus and Carmoviruses. The largest genome other than RCNMV that is packaged by these viruses is TBSV at 4766 nucleotides. This represents a 12% difference between TBSV and an RCNMV RNA-1:RNA-2 virion which packages 5337 nt. An RCNMV virion with a single RNA-1 molecule and two RNA-2 molecules would package 6785 nt which is 42% more RNA than TBSV. Again, given the conservation, it is highly unlikely that RCNMV can physically package any more than around 5800 nucleotides making a virion with more than one copy each of RNA-1 and RNA-2 or 4 copies of RNA-2, impossible.

With two experimental lines of evidence for the co-packaging of RNA-1:RNA-2 as well as the formation of RNA-2 multimers, it seems likely that RCNMV does not strictly employ either Model A or Model B for packaging but rather, a combination of both in the guise of Model C (Fig. 1B). The common denominator for virion populations is the presence of RNA-2. This would point directly to the involvement of RNA-2 as the site for the origin of assembly sequence which is currently being investigated. The packaging arrangement presented here for a multipartite RNA genome within icosahedral virions is unique among both plant and animal viruses.

Materials and methods

Plant inoculations

T7 RNA transcripts of RCNMV RNA-1 and RNA-2 were produced from *Sma*I linearized templates as previously described (Xiong and Lommel, 1991). Uncapped transcripts (5 μl of each genomic RNA) in a total volume of 110 μl of 10 mM sodium phosphate buffer, pH 7.0, were used to inoculate four carborundum-dusted leaves of *N. benthamiana* plants at the 6–8 leaf stage. Inoculated plants were maintained at 18–20 °C under standard glasshouse conditions.

Virus purification

Virions were purified from infected leaf tissue (0.5 g), according to the following protocol. Infected leaf tissue was homogenized in 1 ml of 0.2 M sodium acetate, pH 5.2 containing 0.1% β-mercaptoethanol. The homogenate was centrifuged at 16,250 × *g* for 10 min to pellet cell debris. The supernatant was subsequently filtered through Miracloth and virions were precipitated by the addition of 1/4 volume of 40% PEG 8000 in 1 M NaCl followed by incubation on ice for 30 min. The virions were pelleted by centrifuging at 16,250 × *g* for 10 min and resuspended in 50 μl 10 mM Tris–HCl, pH 6.5.

UV irradiation and heat treatment of virions

For UV irradiation, virion aliquots (50–100 μg) were adjusted to a final volume of 100 μl in 10 mM Tris–HCl, pH 6.5, and chilled on ice for 10 min. The virion sample in a quartz cuvette was deoxygenated by the flow of N_2 gas while being stirred for 30 min. The sample was irradiated with UV radiation (255–300 nm) from a transilluminator (Fotodyne Inc., Hartland, WI) for 20 min (Juzumiene et al., 2001). After irradiation, the virion sample was digested with Proteinase K at 37 °C for 30 min followed by RNA extraction as described below.

For heat treatment, a 30 μg aliquot of virions in 10 mM Tris–HCl, pH 6.5 with 0.1% β-mercaptoethanol was heated at 65 °C for 10 min. The sample was quick cooled on ice for 5 min prior to RNA extraction. Virions were also heated in 5 or 10 °C increments from 35–95 °C to examine the role of temperature on the formation of RNA complexes.

Viral RNA was extracted with phenol–chloroform after addition of SDS to 2% final concentration as described in Lommel et al., (1988).

Virion RNA electrophoresis

For non-denaturing electrophoresis, a 1 μl aliquot of the purified viral RNA was electrophoresed through a 1% agarose gel in Tris–acetate EDTA (TAE) buffer. For semi-denaturing electrophoresis, Tris–borate EDTA (TBE) buffer was used in place of TAE buffer.

For fully denaturing electrophoresis, 1 μl of viral RNA was electrophoresed through a 1% agarose gel containing 1.8% formaldehyde in 1× MOPS (morpholinopropane sulfonic acid, pH 7.0) buffer.

Northern blot hybridization

Electrophoresed viral RNA was blotted to Magnaprobe membranes (GE Osmonics, Minnetonka, MN) by capillary transfer in 5× SSC (sodium chloride–sodium citrate) buffer. The membrane was then subjected to optimal UV crosslinking with the Stratalinker (Stratagene, La Jolla, CA). DNA probes corresponding to the RNA-1 CP open reading frame (ORF) and the RNA-2 MP ORF were labeled with ^{32}P-dCTP via random primer labeling (Rediprime II Random Prime Labeling System, Amersham Biosciences, Piscataway, NJ). The blots were hybridized to the probes overnight at 62 °C in hybridization buffer (7% SDS in 250 mM sodium phosphate, pH 7.0, and 1 mM EDTA). Subsequently, the blots were washed at 60 °C with 5% SDS in 40 mM sodium phosphate, pH 7.0, and 1 mM EDTA followed by a wash with 1% SDS in 40 mM sodium phosphate, pH 7.0, and 1 mM EDTA. The blots were exposed on Phosphoimager screens and visualized with the Storm Gel and Blot Imaging System (Amersham Biosciences).

RNA annealing

Transcripts of RNA-1 and RNA-2 (500 ng each) were subjected to heat treatment individually and in combination at 65 °C in 10 μl dimerization buffer (5 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.5, 40 mM NaCl) followed by slow cooling to room temperature as described in Dirac et al. (2002). Purified virion RNA was also heat treated in a similar manner. The annealed RNAs were analyzed by non-denaturing gel electrophoresis.

Electron microscopy

Purified virions were applied to Formvar-coated copper grids, negatively stained with 2% uranyl acetate and blotted dry. Virion preparations were visualized with a JEM-100 S transmission electron microscope (EM) at 50,000× magnification.

Acknowledgments

We thank Dr. P. Wollenzien for providing laboratory facilities for UV irradiation experiments. We thank Dr. C. L. Hemenway for useful discussion and advice. We also thank Drs. A. Schneemann and N.K. Krishna for helpful analysis and R.H. Guenther for helpful discussions. This work was supported by NSF competitive grant MCB-0077964 to S.A.L. and T.L.S.

References

Ahlquist, P., Dasgupta, R., Kaesberg, P., 1984. Nucleotide sequence of the brome mosaic virus genome and its implications for viral replication. J. Mol. Biol. 172, 369–383.

Choi, Y.G., Rao, A.L., 2003. Packaging of brome mosaic virus RNA3 is mediated through a bipartite signal. Virology 77, 9750–9757.

Darlix, J.L., Gabus, C., Nugeyre, M.T., Clavel, F., Barre-Sinoussi, F., 1990. Cis elements and trans-acting factors involved in the RNA dimerization of the human immunodeficiency virus HIV-1. J. Mol. Biol. 216, 689–699.

Dirac, A.M., Huthoff, H., Kjems, J., Berkhout, B., 2002. Requirements for RNA heterodimerization of the human immunodeficiency virus type 1 (HIV-1) and HIV-2 genomes. J. Gen. Virol. 83, 2533–2542.

Feng, Y.X., Copeland, T.D., Henderson, L.E., Gorelick, R.J., Bosche, W.J., Levin, J.G., Rein, A., 1996. HIV-1 nucleocapsid protein induces "maturation" of dimeric retroviral RNA in vitro. Proc. Natl. Acad. Sci. U.S.A. 93, 7577–7581.

Fu, W., Rein, A., 1993. Maturation of dimeric viral RNA of Moloney murine leukemia virus. J. Virol. 67, 5443–5449.

Fu, W., Gorelick, R.J., Rein, A., 1994. Characterization of human immunodeficiency virus type 1 dimeric RNA from wild-type and protease-defective virions. J. Virol. 68, 5013–5018.

Gould, A.R., Francki, R.I.B., Hatta, T., Hollings, M., 1981. The bipartite genome of red clover necrotic mosaic virus. Virology 108, 499–506.

Guenther, R.H., Sit, T.L., Gracz, H.S., Dolan, M.A., Townsend, H.L., Liu, G., Newman, W.H., Agris, P.F., Lommel, S.A., 2004. Structural characterization of an intermolecular RNA–RNA interaction involved in the transcription regulation element of a bipartite plant virus. Nucleic Acids Res. 32, 2819–2828.

Hamilton, R.I., Tremaine, J.H., 1996. Dianthoviruses: properties, molecular biology, ecology, and control (Chapter 10). In: Harrison, B.D., Murant, A.F. (Eds.), The Plant Viruses, Polyhedral Virions and Bipartite RNA Genomes, vol. 5. Plenum Press, New York, NY, pp. 251–282.

Hollings, M., Stone, O.M., 1977. Red clover necrotic mosaic virus. CMI/AAB Descript. Plant Viruses (181) (4 pp.).

Juzumiene, D., Shapkina, T., Kirillov, S., Wolenzien, P., 2001. Short-range RNA-RNA crosslinking methods to determine rRNA structure and interactions. Methods 25, 333–343.

Krishna, N.K., Schneemann, A., 1999. Formation of an RNA heterodimer upon heating of nodavirus particles. J. Virol. 73, 1699–1703.

Lane, L.C., 1977. Brome mosaic virus. CMI/AAB Descript. Plant Viruses (180) (4 pp.).

Laughrea, M., Jette, L., 1996. Kissing-loop model of HIV-1 genome dimerization: HIV-1 RNAs can assume alternative dimeric forms, and all sequences upstream or downstream of hairpin 248–271 are dispensable for dimer formation. Biochemistry 35, 1589–1598.

Laughrea, M., Jette, L., Johnson, M., Kleiman, L., Liang, C., Wainberg, M.A., 1997. Mutations in the kissing loop hairpin of human immunodeficiency type 1 reduce viral infectivity as well as genomic RNA packaging and dimerization. J. Virol. 71, 3397–3406.

Lommel, S.A., Weston-Fina, M., Xiong, Z., Lomonossoff, G.P., 1988. The nucleotide sequence and gene organization of red clover necrotic mosaic virus RNA-2. Nucleic Acids Res. 16, 8587–8602.

Mayo, M.A., Harrison, B.D., Murant, A.F., Barker, H., 1973. Cross-linking of RNA induced by ultraviolet irradiation of particles of raspberry ringspot virus. J. Gen. Virol. 19, 155–159.

Mujeeb, A., Clever, J.L., Billeci, T.M., James, T.L., Parslow, T.G., 1998. Structure of the dimer initiation complex of HIV-1 genomic RNA. Nat. Struct. Biol. 6, 432–436.

Muriaux, D., De Rocquigny, H., Roques, B.P., Paoletti, J., 1996a. NCp7 activates HIV-1Lai RNA dimerization by converting a transient loop–loop complex into a stable dimer. J. Biol. Chem. 271, 33686–33692.

Muriaux, D., Fosse, P., Paoletti, J., 1996b. A kissing complex together with a stable dimer is involved in the HIV-1 Lai RNA dimerization process in vitro. Biochemistry 35, 5075–5082.

Newman, J.F.E., Brown, F., 1977. Further physicochemical characterization of Nodamura virus. Evidence that the divided genome occurs in a single component. J. Gen. Virol. 38, 83–95.

Osman, T.A.M., Buck, K.W., 1991. Detection of the movement protein of red clover necrotic mosaic virus in a cell wall fraction from infected *Nicotiana clevelandii* plants. J. Gen. Virol. 72, 2853–2856.

Patton, J.T., Spencer, E., 2000. Genome replication and packaging of segmented double-stranded RNA viruses. Virology 277, 217–225.

Qiao, X., Qiao, J., Mindich, L., 1997. Stoichiometric packaging of the three genomic segments of double-stranded RNA bacteriophage 6. Proc. Natl. Acad. Sci. U.S.A. 94, 4074–4079.

Sakuragi, J., Ueda, S., Iwamoto, A., Shioda, T., 2003. Possible role of dimerization in human immunodeficiency virus type 1 genome RNA packaging. J. Virol. 77, 4060–4069.

Selling, B.H., Rueckert, R.R., 1984. Plaque assay for black beetle virus. J. Virol. 51, 251–253.

Sit, T.L., Vaewhongs, A.A., Lommel, S.A., 1998. RNA-mediated trans-activation of transcription from a viral RNA. Science 281, 829–832.

Tremaine, J.H., Ronald, W.P., 1976. Differential effects of sodium dodecyl sulfate on strains of carnation ringspot virus. J. Gen. Virol. 30, 299–308.

van Regenmortel, M.H.V., et al., 2000. Virus taxonomy: classification and nomenclature of viruses. Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, CA.

Whirl-Carrillo, M., Gabashvili, I.S., Bada, M., Banatao, D.R., Altman, R.B., 2002. Mining biochemical information: lessons taught by the ribosome. RNA 8, 279–289.

White, K.A., 2002. The premature termination model: a possible third mechanism for subgenomic mRNA transcription in (+)-strand RNA viruses. Virology 304, 147–154.

Xiong, Z., Lommel, S.A., 1989. The complete nucleotide sequence and genome organization of Red clover necrotic mosaic virus RNA-1. Virology 171, 543–554.

Xiong, Z., Lommel, S.A., 1991. Red clover necrotic mosaic virus infectious transcripts synthesized in vitro. Virology 182, 388–392.

Xiong, Z., Kim, K.H., Giesman-Cookmeyer, D., Lommel, S.A., 1993a. The roles of the Red clover necrotic mosaic virus capsid and cell-to-cell movement proteins in systemic infection. Virology 192, 27–32.

Xiong, Z., Kim, K.H., Kendall, T.L., Lommel, S.A., 1993b. Synthesis of the putative red clover necrotic mosaic virus RNA polymerase by ribosomal frameshifting in vitro. Virology 193, 213–331.

Zavriev, S.K., Hickey, C.M., Lommel, S.A., 1996. Mapping of the red clover necrotic mosaic virus subgenomic RNA. Virology 216, 407–410.