ABSTRACT

BASNAYAKE, VERONICA ROSHANI. Identification and characterization of the Red clover necrotic mosaic virus origin of assembly sequence. (Under the direction of Dr. Steven A. Lommel).

Red clover necrotic mosaic virus (RCNMV) is a single-stranded positive-sense RNA plant virus of the Dianthovirus genus, family Tombusviridae. Each RCNMV virion contains 180 subunits of the 37 kDa capsid protein (CP) forming a non-enveloped, isometric particle of T=3 quasi symmetry, 30-35 nm in diameter. The RCNMV genome consists of two RNAs, RNA-1 and RNA-2. RNA-1 codes for three proteins: i) p88, the RNA-dependent RNA polymerase, ii) p27, the replicase related protein and iii) the CP. RNA-2 is monocistronic and codes for the movement protein (MP).

Currently, the RNA complement within RCNMV virions has not been determined. Density gradient centrifugation data has suggested the probability of a single type of particle, whereas the viral RNA profile from virions suggests otherwise. My thesis research has determined the RNA content within RCNMV virions after exposure to either heat or UV-irradiation. Both treatments result in the formation of a stable RNA-1: RNA-2 heterodimer. This leads to the conclusion that RCNMV virions co-package RNA-1 and RNA-2. Upon either treatment, RNA-2 multimers are also observed, suggesting the presence of particles packaging RNA-2 exclusively. These
observations suggest that the RCNMV virion population consists of two distinct types of particles having similar densities.

Based on the above RNA complement findings, I proceeded to delineate the specific viral sequences that determine the RNA content of an RCNMV virion. During virion assembly, the RCNMV CP must be able to distinguish and package both RNA-1 and RNA-2 while excluding heterologous host RNAs present in the cell. Viral CP subunits recognize specific cognate genomic sequences and/or structures that are unique to each viral genome. These are designated as origin of assembly sequences or packaging signals. To elucidate the assembly mechanism of RCNMV, I searched for the presence of distinct packaging signals on each of the genomic RNAs. Various constructs of RNA-1 and RNA-2 were tested for their assembly efficiencies in vivo using a plant assay system. While it has been previously demonstrated that RNA-1: RNA-2 base pairing directs the synthesis of the CP subgenomic RNA (sgRNA) from RNA-1 via the trans-activating (TA) element on RNA-2, it was not determined whether this interaction had a role in assembly. I have found that RNA-1 does not have the ability to package by itself given sufficient amounts of CP. Also, the CP sgRNA is not encapsidated into RCNMV virions. RNA-2 appears to play the major role in directing RCNMV assembly. My results indicate that a 209 nt sequence within the MP open reading frame on RNA-2 (containing the TA element) directs RCNMV assembly. Deletion mutagenesis of RNA-2 to delimit the packaging signal proved that the 34-nucleotide TA element was the origin of assembly. As further proof, expression of the TA element from a Tomato bushy stunt
virus vector directed the co-packaging of RCNMV RNA-1 into virions proving it to be essential for RCNMV assembly. Deletion mutagenesis also revealed that RCNMV has an RNA packaging size requirement for production of stable virions.

Based on all of the above observations, I propose the following model for the RCNMV packaging mechanism: the base pairing interaction of the TA element with RNA-1 initiates CP production and enables the formation of an RNA-1: RNA-2 heterodimer. This dimer formation allows the co-packaging of both RNAs into a single virion via the packaging signal on RNA-2. The discreet packaging signal on RNA-2 also allows the formation of some virions containing solely RNA-2.
IDENTIFICATION AND CHARACTERIZATION OF THE RED CLOVER NECROTIC MOSAIC VIRUS ORIGIN OF ASSEMBLY SEQUENCE

by

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A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

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I was born and educated in Colombo, the capital of the tropical island Sri Lanka. I started my primary education at St. Joseph’s Convent School and later joined Visakha Vidyalaya for my secondary education. I finished high school and started my undergraduate studies at the University of Colombo. I graduated with first class honors in Botany in 1997. While in college, the subject of plant pathology caught my attention and I decided to continue on with my graduate studies. With that in mind I applied to graduate school at University of Delaware, Newark, DE and was accepted in to the program and made the journey to the USA. I graduated with an MS degree in Plant Science in May 2000 and the project was to determine the host response to a plant virus and this encouraged me to further my education in plant viruses. I joined the Lommel lab at North Carolina State University in August 2000. Coming to North Carolina has been a wonderful experience for me as I was fortunate to meet some lovely people. More importantly, it gave me the chance to experience a breathtakingly beautiful part of the US.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>list of tables</td>
<td>vii</td>
</tr>
<tr>
<td>list of figures</td>
<td>viii</td>
</tr>
<tr>
<td>list of abbreviations</td>
<td>x</td>
</tr>
</tbody>
</table>

## Chapter 1

**Red Clover Necrotic Mosaic Virus: A Review**

- **Introduction** ......................................................... 1
- **Symptomatology** ..................................................... 4
- **Cytopathology** ....................................................... 4
- **Transmission** .......................................................... 5
- **Control** .................................................................. 6
- **Physical, chemical and biochemical properties** ....................... 6
  1. Purification of RCNMV ............................................ 6
  2. Electron microscopy ............................................... 7
  3. Virion properties ................................................... 7
- **Serology** .................................................................... 8
- **Genome properties** .................................................. 9
- **RCNMV RNA-1** ........................................................ 9
- **RCNMV RNA-2** ........................................................ 10
- **Ribosomal frameshifting** ........................................ 12
- **Replication** ............................................................ 13
- **Trans-activation** .................................................... 15
- **Movement** .................................................................. 17
- **The research project** .............................................. 23
- **References** ................................................................ 33

## Chapter 2

**Virus Packaging Signals: A Review**

- **Introduction** ............................................................ 44
- **Animal virus OASs** .................................................. 45
  - **Retroviruses (family Retroviridae)** .......................... 49
  - **Human immunodeficiency virus -1**                     54
  - **Alphaviruses (family Togaviridae)** ........................ 59
  - **Mouse hepatitis virus (family Coronavirusidae)** ....... 61
- **Hepadnaviruses (family Hepadnaviridae)** ...................... 63
  - **Adenoviruses (family Adenoviridae)** ....................... 64
  - **Flock house virus (family Nodaviridae)** .................. 65
- **Plant Virus OASs** ................................................... 66
  - **Tobacco mosaic virus** ............................................ 66
  - **Papaya mosaic virus (family Potexviridae)** ................. 69
  - **Tobacco vein mottling virus (family Potyviridae)** ....... 69
  - **Peanut clump virus** .............................................. 70
  - **Turnip crinkle virus (family Tombusviridae)** ............ 71
  - **Turnip yellow mosaic virus** ................................... 72

iv
**Cowpea chlorotic mottle virus** *(family Bromoviridae)* ..................73

**Brome mosaic virus** *(family Bromoviridae)* ...........................74

Summary .............................................................................................................75

References .......................................................................................................... 90

---

**Chapter 3**  
The Packaging Scheme of *Red Clover Necrotic Mosaic Virus* Genomic RNAs

Introduction ..........................................................................................................106

Materials and methods.........................................................................................110

Results .................................................................................................................113

An RNA-1: RNA-2 heterodimer is produced upon UV irradiation of RCNMV virions .................................................................113
An RNA-1: RNA-2 heterodimer is also formed upon heat treatment of virions .................................................................................115
Formation of the RNA-1: RNA-2 heterodimer is temperature dependent ........................................................................................116
The RNA-1: RNA-2 heterodimer is formed within virions by means of a non-covalent interaction .................................................116
RCNMV RNA does not form dimer complexes upon heating in vitro ..................................................................................117

Discussion ...........................................................................................................118

Acknowledgements ..............................................................................................124

References .......................................................................................................... 133

---

**Chapter 4**  
The *Red Clover Necrotic Mosaic Virus* Origin of Assembly: A Third Function for the RNA-2 Trans-Activator ...

Introduction ..........................................................................................................139

Materials and methods.........................................................................................144

Results .................................................................................................................149

RCNMV sgRNA is not packaged into RCNMV virions .................................149
RNA-1 does not package into virions in the absence of RNA-2 .................150
A 209 nt region of RCNMV RNA-2 directs encapsidation of heterologous TBSV RNA in the presence of RCNMV CP ..................151
Refining the minimal OAS on RCNMV RNA-2 by deletion mutagenesis ......152
TA loop mutation does not disrupt OAS function ........................................153
The RCNMV TA element functions as an OAS in a heterologous context 154
Encapsidation of heterologous RNAs defines an upper packaging size limit 154

Discussion ...........................................................................................................155
Acknowledgements .................................................................................................................. 165
References ................................................................................................................................. 180

Chapter 5  Red Clover Necrotic Mosaic Virus Particles Consist of Both Native and Expanded Forms ................................................................................................................. 186

Introduction ............................................................................................................................... 188
Materials and methods ............................................................................................................. 192
Results ........................................................................................................................................ 195
Optiprep™ and CsCl density gradient separation yields swollen and native forms of RCNMV virions ................................................................. 195
Differential density of the RCNMV virions is not due to a modification of CP ............................ 196
Virion RNA composition of the swollen and native RCNMV virions ........................................ 197
Discussion .................................................................................................................................... 197
References ..................................................................................................................................... 205
LIST OF TABLES

Chapter 4

Table 1. Sequences of the oligonucleotides used for the synthesis of RNA-2 deletion mutants .................................................................166
LIST OF FIGURES

Chapter 1

Figure 1. Symptoms caused by RCNMV on *Nicotiana benthamiana*..............27
Figure 2. The electron microscopic image of RCNMV virions.........................28
Figure 3. The genome organization of RCNMV............................................29
Figure 4. The 3' termini of RCNMV RNA-1 and RNA-2..............................30
Figure 5. Trans-activation and CP sgRNA synthesis..................................31
Figure 6. The structure of the TBSV particle architecture............................32

Chapter 2

Figure 1. Predicted secondary structure for *Rous sarcoma virus* encapsidation (Ψ) signal.................................................................77
Figure 2. The secondary structure model of the *Avian sarcoma leukemia virus* packaging region............................................................78
Figure 3. Secondary structure of the *Moloney murine leukemia virus* encapsidation signal (Ψ).................................................................79
Figure 4. HIV-1 packaging signal (Ψ) ...........................................................80
Figure 5. Alternative foldings of the HIV-1 leader .......................................81
Figure 6. Secondary structure of the *Bovine leukemia virus* discontinuous encapsidation signal .......................................................82
Figure 7. Predicted secondary structure of the *Mouse hepatitis virus* 69-nucleotide packaging signal.......................................................83
Figure 8. The predicted secondary structure of *Hepatitis B virus* encapsidation (ε) signal ........................................................................84
Figure 9. Secondary structures of duck and heron hepatitis B virus encapsidation signals.................................................................85
Figure 10. Predicted structure of the packaging signal of *Flock house virus*.........................................................................................86
Figure 11. The *Tobacco mosaic virus* origin of assembly..............................87
Figure 12. Secondary structure of the *Turnip crinkle virus* essential element for packaging .................................................................88
Figure 13. The *Turnip yellow mosaic virus* 5' leader region with two hairpins containing protonatable internal loops ..........................89

Chapter 3

Figure 1. *Red clover necrotic mosaic virus* genome and various possibilities for packaging the genomic RNAs.................................125
Figure 2. UV crosslinking of RCNMV virions .............................................126
Figure 3. Gel electrophoresis of virion RNA purified from heated
and unheated RCNMV virions ..........................................................127

Figure 4. Denaturing gel electrophoresis and northern analysis
of heat treated and UV-crosslinked RCNMV virion RNA .................128

Figure 5. Temperature range for RCNMV RNA complex
formation and effects of heating on virion morphology ...................129

Figure 6 RNase T1 treatment of heated and unheated virions ..........130

Figure 7. Heating of RCNMV transcripts and virion purified RNA ...131

Figure 8. A comparison of the TA, TABS and TABS$_M$ sequences ....132

Chapter 4

Figure 1. Red clover necrotic mosaic virus genome organization .......167
Figure 2. RCNMV CP sgRNA is not packaged into virions ...............168
Figure 3. RCNMV RNA-1 does not contain a discrete OAS ...............169
Figure 4. RCNMV virion assembly directed by the TBSV sgRNA
containing a RCNMV RNA-2 OAR fragment ..............................170
Figure 5. Virion formation of the RNA-2 deletion mutants ...............171
Figure 6. The role of TA terminal loop in assembly .........................175
Figure 7. The RCNMV RNA-2 TA is the OAS ..................................176
Figure 8. RNA size requirement for RCNMV packaging ..................177
Figure 9. An RCNMV assembly model .............................................179

Chapter 5

Figure 1. Separation of swollen and native RCNMV virions ..........202
Figure 2. CP of swollen virions do not appear to be modified ..........203
Figure 3. RCNMV viral RNA purified from native and swollen virions ...204
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARM</td>
<td>arginine-rich motif</td>
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<td>ASLV</td>
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Chapter 1

Red Clover Necrotic Mosaic Virus: A Review
Red Clover Necrotic Mosaic Virus: A Review

Introduction

Red clover necrotic mosaic virus (RCNMV) was first described by Musil and Matisova (1967) in Czechoslovakia based on symptoms in red clover (Trifolium pratense) and alfalfa (Medicago sativa). The virus was isolated and distinguished from other clover infecting viruses by host range, physical properties, electron microscopy and serological studies (Musil, 1969 a and b). RCNMV is a positive-sense single-stranded RNA plant virus with isometric particles 30-35 nm in diameter. Different isolates and strains have been reported from northern Europe (primarily England, Czechoslovakia and Sweden), Canada, Australia and New Zealand. RCNMV naturally occurs in red clover, sweet clover, white clover and alfalfa in field crops or in pastures (Hiruki, 1987). The virus is easily mechanically transmissible to a wide range of herbaceous hosts although the natural host range is limited. They can systemically infect a number of species in the host families Solanaceae, Leguminosae, Cucurbitaceae and Asteraceae, and others locally. Nicotiana clevelandii, N. benthamiana and Phaseolus vulgaris are suitable propagation species while Chenopodium quinoa is a good local lesion host. There are no known vectors for RCNMV. RCNMV is transmitted through soil (Hiruki, 1987, Hollings and Stone, 1977), but not seed-borne (Hollings and Stone, 1977).

Taxonomically RCNMV resides in the family Tombusviridae, which is a diverse family of icosahedral viruses with single-stranded, positive-sense RNA genomes. The family is represented by the genera Tombusvirus, Carmovirus,
Necrovirus, Dianthovirus, Machlomovirus, Panicovirus, Avenavirus and Aureovirus. The members of the family share two important features: the RNA-dependent RNA polymerase is phylogenetically conserved in all members and the viruses are primarily transmitted in soil. All members of Tombusviridae contain a single genomic RNA, except dianthoviruses. Dianthoviruses are distinguished from the other members of the family due to the segmented nature of their genome. Members of Tombusviridae comprise of Capsid proteins (CP) with two distinct phylogenetic origins. One group is formed with CP with a molecular weight of 37-48 kDa containing protruding domains and the other lacking protruding domains with a molecular weight of 27-29 kDa (Lommel et al., 2000). The members of the genus Dianthovirus, created in 1981 by the ICTV (Matthews, 1982), contain two non-homologous single-stranded positive-sense RNAs. RCNMV produces T=3 icosahedrons composed of 180 units of a 37 kDa CP. The dianthovirus CP is grouped within the CP with protruding domains (Aureusvirus, Avenavirus, Carmovirus, Dianthovirus, and Tombusvirus) giving rise to virions with a granular outline. The phylogenetic group lacking the protruding domain (Machlomovirus, Panicovirus, and Necrovirus) produces virions with a smooth outline. There are three known members of the genus: Carnation ringspot virus (CRSV), the type member, RCNMV and Sweet clover necrotic mosaic virus (SCNMV). Furcrea necrotic streak virus (FNSV), which causes a necrotic streak of fique (Furcrea macrophylla and F. cabuya) is serologically related to RCNMV and could be a fourth possible species (Morales et al., 1992). Dianthoviruses do not cause severe crop losses. Their distribution is widespread throughout the temperate regions except for FNSV, which
is tropical in range. CRSV spread is worldwide whereas RCNMV is commonly found in temperate Europe, Canada, Australia and New Zealand. SCNMV has only been found in Alberta, Canada.

Symptomatology

Symptoms on infected red clover include necrotic lesions, severe leaf mottle, necrosis of the veins, distortion of leaves and moderate to severe stunting (Fig. 1). The symptoms are more pronounced in the winter, but masked in hot weather, particularly in newly infected plants. Inoculated red clover or Nicotiana species primarily show local necrotic ringspot lesions. Although a wide variation in symptomatology has been reported for RCNMV, Okuno et al. (1983) observed that in controlled growth chambers with temperature maintained at 17 -26°C plants showed reproducible symptoms. The virus is present in leaves, stems and roots and has been observed in soil surrounding the rootstock.

Cytopathology

Virions have been observed scattered in the cytoplasm and vacuole of N. clevelandii leaf mesophyll cells (Francki et al., 1985). Large aggregates of virions as well as tubular inclusions occur in CRSV-infected sweet william and cowpea (Weintraub et al., 1975). RCNMV or SCNMV do not frequently produce inclusions, however, amoeboid inclusion bodies have been observed in less than 5% of the RCNMV-infected N. benthamiana cells (Hollings and Stone, 1977). Virions have
been associated with microtubules in cells infected with both CRSV and SCNMV, but not observed with RCNMV (Francki et al., 1985).

**Transmission**

Contact transmission and soil-borne transmission without the aid of vectors seem to be the natural modes of transmission for RCNMV. Dianthovirus virions are readily released from root cells into soil water where they may remain infective for months (Hollings and Stone, 1977; Kegler and Kegler, 1981; Hiruki, 1986). The remarkably stable nature of the particles enables the viruses to remain infective for long time periods. RCNMV is more stable than other dianthoviruses and is known to be more resistant to swelling and proteolysis of the virion (Gould et al., 1981; Hamilton and Tremaine, 1996). Although dianthoviruses have been reported to be transmitted by longidorid nematodes they have not been confirmed (Fritzsche, 1968; Fritzsche and Schmelzer, 1967; Kleinhempel et al., 1980). Transmission of RCNMV was enhanced 2- to 20-fold in the presence of the Chytridiomycete, *Olpidium brassicae* suggesting that the fungal vector might be involved in its transmission (Gerhardson and Insunza, 1979). MacFarlane (1982) reported that *O. bornovanus* (Sahtiyanci), which is more prevalent in Britain, transmitted RCNMV to clover. Although fungal and/or nematode vectors have been thus far suggested as modes of dianthovirus transmission, it is likely that the contamination of soil water with infective virions may have been responsible for the infections. Further work is required for proof of vector transmission and it is possible that nematode and fungal
vectors predispose plant roots to infection by creating wounds for initial virus infection.

**Control**

In vegetatively propagated carnation and fruit trees (in the case of CRSV) control of virus has been achieved by thermotherapy (Brierley, 1964) or meristem-tip culture (Stone, 1968; Kowalska, 1974). Usage of such propagation techniques coupled with constant monitoring with enzyme linked immunosorbent assay (ELISA) should eradicate virus infections (Lommel et al., 1983). Control of RCNMV could pose greater problems due to its broader host range however dianthovirus plant diseases have not so far posed economical concerns.

**Physical, chemical and biochemical properties**

1. **Purification of RCNMV**

High viral concentrations are achieved in RCNMV infected plants. Local lesions on *N. benthamiana* leaves appear 2-3 days after inoculation of the virus and systemic symptoms appear 4-5 days post inoculation. Virus titer is largely dependent on environmental conditions and plants grown at 18-20°C and 16 hours/day illumination at a light intensity of ~5000 lux consistently produce high virus concentrations, approximately 100 μg of virus per gram of leaf tissue (Hollings and Stone, 1977). Virus purification has been carried out in 0.1 M phosphate buffer, pH
7.4 (Gould et al., 1981) and is also successfully achieved by 0.2 M Tris-acetate buffer, pH 5.2. RCNMV virions appear to be most stable at pH 6.8.

2. Electron microscopy

Virion morphology of the dianthoviruses is very similar to other members of *Tombusviridae* family with similar phylogenetically conserved CP having a protruding domain. RCNMV virions exhibit a spherical morphology with a granular surface (Fig. 2). Diameter for RCNMV ranges from 27-35 nm (Hiruki et al., 1984b; Hollings and Stone, 1965; Bowen and Plumb, 1979; Kuhne and Eisbein, 1983).

3. Virion properties

RCNMV virions sediment as a single component with a sedimentation coefficient (*S*<sub>20,w</sub>) at about 130-135 S at, pH 5.0 (Hollings and Stone, 1970, 1977). Virus concentration, age of the virus preparation, virus purification methods and the pH of the virus preparation are known to affect the sedimentation coefficient (Hiruki, 1987). RCNMV has a buoyant density of approximately 1.363 g/cm³ in cesium chloride. The Swedish strain of RCNMV has an extra component at 1.356 g/cm³ (Hollings and Stone, 1977). RCNMV genome is encapsidated by 180 subunits of 37 kDa capsid protein to form a virion of T=3 symmetry (Lommel, 1983; Morris-Krsinich et al., 1983). The capsid protein subunit consists of approximately 339 amino acids and the amino acid composition of different RCNMV strains is basically similar (Hamilton and Tremaine, 1996). The RNA complement within the virion was estimated to be around 20% of total particle weight. RCNMV packaging complement
has thus far not been determined. CRSV particles swell slowly when pH is changed from 5.0 to 7.5 (Tremaine and Ronald, 1976). Treatment with EDTA accelerates this swelling but Mg$^{2+}$ or Ca$^{2+}$ ions reverse it (Tremaine et al., 1976). However, RCNMV which is more stable than CRSV (Hamilton and Tremaine, 1996) does not seem to swell at pH 7.0 but proteolysis of virions samples has been observed in storage. RCNMV-Can strain when treated with pH 7.5 for 1 hour exhibited swelling in 20% of particles. Half of the virions were dissociated when treated with 1% SDS. RCNMV completely dissociated when pH was elevated to 8.25. Capsid protein subunits reassembled in the presence of the nucleating agent sodium dextran sulfate to form T=1 and T=3 virions, which could be readily distinguished by electron microscopy (J.H. Tremaine and W.P. Ronald, unpublished results, Hamilton and Tremaine, 1996).

**Serology**

Serological studies of RCNMV initially distinguished three serotypes, A (RCNMV-TpM34) and B (RCNMV-TpM48) from Czechoslovakia (Musil, 1969b), and C (RCNMV-Sw) from Sweden (Gerhardson and Lindsten, 1973). Serological analysis by intragel cross-absorption experiments revealed that the Canadian strain (RCNMV-Can) belongs to serotype B and Australian (RCNMV-Aus) and English (RCNMV-Eng) strains belong to serotype D (Rao et al., 1987). RCNMV-Aus and RCNMV-Eng were serologically indistinguishable and did not react with the antisera of any of the three serotypes of the virus. There was no cross-reaction between RCNMV and antisera to CRSV or SCNMV (Hiruki et al., 1984a) in direct, double
antibody sandwich ELISA, however, indirect ELISA gave weak cross-reactions with them (Van Regenmortel and Burckard, 1980). The isoelectric points of RCNMV strains are in the range of pH 4.75-5.1 (Pappu and Hiruki, 1989).

**Genome properties**

The bipartite nature of RCNMV (Fig. 3) was first described by Ragetli and Elder (1977) who observed two distinct RNA species in density gradient centrifugation of the products of sodium dodecyl sulfate (SDS)-denatured virus particles. Although this observation was attributed to clover primary leaf necrosis virus, it is now considered to be the RCNMV-Ca strain based on host range, serology, nucleic acid and capsid mobilities (Rao and Hiruki, 1985; Hamilton and Tremaine, 1996). The RNA-1 of the dianthoviruses are similar in size (CRSV type - 3756 bases, RCNMV-Aus- 3889 and SCNMV-59 alfalfa isolate- 3876). The RNA-2 molecules however, differ slightly in size (CRSV-1394 bases, RCNMV-Aus- 1448 and SCNMV-59-1449). The complete sequence of RCNMV RNA-1 and RNA-2 has been published (Xiong and Lommel, 1989; Lommel et al., 1988). Both RNA-1 and RNA-2 are required for infectivity.

**RCNMV RNA-1**

RNA-1 consists of three open reading frames (ORF) (Fig. 3). The 5’ terminal ORF encodes a 27 kDa polypeptide (p27) and an 88 kDa (p88) polypeptide, which is translated as an extension of p27 due to ribosomal frameshift near the stop site of p27 ORF. The 3’ terminal ORF encodes the 37-kDa capsid protein (CP) (Fig. 3A)
(Xiong and Lommel, 1989). The p88 polypeptide is the viral RNA-dependent RNA polymerase (RdRp) and p27 is a replicase related protein. The 3' terminal CP gene is expressed *in vivo* from a subgenomic RNA. The genomic RNA-1 has a 5' 122-nucleotide leader followed by the p27 ORF initiating at nucleotide 123 and terminating at an amber terminator at 831. The polypeptide p88 extends the p27 and terminates at 2423. The 3' terminal CP ORF initiates at 2427 and terminates at 3444. There is a 445-nucleotide 3' terminal non-coding region.

RCNMV RNA-2

The RNA-2 molecule is monocistronic and codes for a 35 kDa movement protein (MP; Lommel et al., 1988). The first initiation codon of the ORF of MP is at nucleotide 80 and ends at nucleotide 1030 (Fig 3.B). The C-terminal region of MP is required for the movement function. The MP C-terminus shares some identity with bromoviruses, *Brome mosaic virus* and *Cowpea chlorotic mottle viruses*, at the sequence level (Lommel et al., 1988; Allison et al., 1989). Computer translation of the RNA-2 revealed the presence of another ORF downstream of the MP ORF, with the start position at 1224 and ending at 1358. This has the probability to encode a 4.8 kDa polypeptide, although none has been observed *in vitro*. Subgenomic RNA corresponding to it has not been detected either (Lommel et al., 1988) and deleting this ORF results in no change in phenotype.

Folding of the RNA-1 5' leader sequence does not reveal significant secondary structure formation and this is typical for plant viruses. The 5' leaders of the two RNAs share little sequence homology and one plus strand replication
promoter. The 5' RNA-2 leader sequence is 79 nucleotides in length and is in the range observed for other spherical RNA plant viruses (Davies and Hull, 1982). RNA-2 has high adenine content (44% of the nucleotides). The 3' termini of both RNA-1 and RNA-2 on the other hand contain two highly homologous domains (Fig. 4). The first homologous domain extends from RNA-1 position 3817 to 3852 and RNA-2 from 1315 to 1346 nucleotides. The second homologous region on RNA-1 is from 3863 to 3889 and on RNA-2 from 1422 to 1448. The 27 nucleotide terminal homologous sequence forms a highly stable stem loop. The 3' non-coding regions of viral RNAs naturally serve as replicase recognition sites with complex secondary structures or sequences (Dreher et al., 1984; Bujarski et al., 1986). Turner and Buck (1999) showed that the 5' leader and most of the 3' untranslated region of RNA-2 were required for replication and that the 3' proximal stem loop was indispensable for replication. Most recently, an element on RNA-2 was identified as an essential cis-replicating RNA element of RNA-2 (Tatsuta et al., 2005). The 3' termini for both RNA-1 and RNA-2 are longer than the typical 150- to 250-base non-coding regions for other viruses (Davies and Hull, 1982). There is the possibility that there are extra ORFs in these regions for which the corresponding polypeptides have not been identified.

RCNMV genomic RNAs lack a 5' cap structure (m7GpppN; Mizumoto et al., 2003). The eukaryotic cap structure together with the 3' poly (A) tail act as binding sites for the translational machinery for initiation of translation. In the absence of these standard translation elements secondary sequences functions as translational enhancers. Barley yellow dwarf virus (BYDV) and Tomato bushy stunt virus (TBSV),
both lack a 5’ cap and a poly (A) tail, however, the genomic RNA consists of translation enhancer sequences in the 3’ untranslated region that confer cap-independent translation (Wang et al., 1997; Wu and White, 1999). Since RCNMV RNAs are uncapped and without 3’ polyadenylation, it was assumed that they contained translation enhancer sequences within their genomes. Recently RCNMV RNA-1 was demonstrated to contain a translation element in the 3’ untranslated region (nts 3596 to 3732 (Mizumoto et al., 2003). A stem loop within this translation element that is conserved among the dianthoviruses appears to be the main component of the cap-independent translation. This stem loop was shown to be nearly identical to the translation enhancers identified from BYDV and *Tobacco necrosis virus*.

**Ribosomal frameshifting**

Ribosomal frameshifting is an approach for regulating protein expression at the translational level that is utilized by eukaryotic organisms. This phenomenon was first observed in *Rous sarcoma virus* synthesis of viral reverse transcriptase (Jacks and Varmus, 1985; Jacks et al., 1988). A similar mechanism operates in the synthesis of RNA polymerases of BYDV (Veidt et al., 1988) and *Potato leafroll luteovirus* (Prufer et al., 1992) as well as coronaviruses (Brierley et al., 1989) and other retroviruses (Varmus, 1988). A slippage model of -1 ribosomal frameshifting was proposed as a specific heptanucleotide where the ribosome slippage occurs (Jacks et al., 1998). The shifty heptanucleotides conform to a generalized sequence of X XXY YYZ (X=A,G, or U; Y=A or U; Z=A,C, or U). After slippage into the –1 frame, partial or full codon-anticodon base pairing needs to be maintained for the
frameshift event and to ensure continuity of translation. An RNA stem loop structure immediately downstream of this shifty heptanucleotide is also believed to be facilitating the frameshift event (Rice et al., 1985; Brierley et al., 1989; Prufer et al., 1992). The shifty heptanucleotide causes tRNA slippage during translation and the stable secondary stem loop downstream is believed to stall or slow ribosome migration increasing the probability of slippage leading to the -1 frameshift event (Somogyi et al., 1993). The frameshift mechanism of the heptanucleotide RCNMV was established by point and frameshift mutations in in vitro synthesis of 88 kDa polymerase (Kim and Lommel, 1994; Xiong et al., 1993b). The shifty heptanucleotide of RCNMV has similarities to those of BYDV, Rous sarcoma virus and Mouse mammary tumor virus. The RCNMV shifty heptanucleotide was functionally interchangeable with many plant shifty heptanucleotide signals but not with those of mammalian signals. RCNMV was able to revert the shifty signal mutants back to the wild type. Similar structures have been identified in RNA-1 of CRSV and SCNMV that could be involved in a frameshift mechanism (Ryabov et al., 1994). An RNA secondary structure, a stem loop element downstream of the shifty heptanucleotide is implicated to assist frameshift event similar to others. Mutations that destabilized this stem loop did not function in vitro, and they were unable to replicate in vivo (Kim and Lommel, 1998).

**Replication**

RCNMV replicates rapidly in host plants kept at 22°C and the virus titer reaches the peak in about 2-4 days. Virus titer in plants is greatly affected by the
ambient temperature in which they are grown. Since RCNMV produces a high virus titer in *Nicotiana* species, it is well suited for assembly and replication studies. RCNMV replication occurs in two stages: the RNA negative-strand is synthesized from the parental positive-strand genomic RNA and, which in turn acts as a template to synthesize progeny positive-strand RNA. Virus encoded RdRp catalyses the synthesis of progeny RNA molecules from nucleoside triphosphate substrates. Koonin (1991), proposed three phylogenetic supergroups of RdRp based on sequence similarities of conserved amino acid motifs. Supergroup I include virus genera, *Picornavirus, Nodavirus, Comovirus, Nepovirus, Potyvirus, Bymovirus, Sobemovirus* and others. Members of *Carmovirus, Necrovirus, Tombusvirus,* and *Hepatitis C virus* belong to supergroup II. Supergroup III consists of *Tobamovirus, Tobravirus, Hordeivirus, Alfamovirus, Bromovirus, Cucumovirus, Alphavirus, and Potexvirus* among others. The RCNMV 88 kDa polypeptide (*p88*) contains the GDD (Glycine: Aspartic acid: Aspartic acid) motif which is conserved in all viral RNA polymerases (Koonin, 1991). Based on the high degree of sequence similarity, RCNMV RdRp is grouped in supergroup II together with other members of family *Tombusviridae* and *Luteoviridae* (Koonin and Dolja, 1993; Koonin, 1991). RCNMV RdRp is template specific and therefore only able to utilize RCNMV RNAs but not RNAs of taxonomically different viruses (Bates et al., 1995). RCNMV 27 kDa polypeptide (*p27*) was found to be a component of the viral RdRp purified from *N. clevelandii* suggesting that it could be playing a role in replication such as template recognition or as a scaffold for other replication proteins in the replication complex.
Recently it was established that positive-strand RNA viruses replicate in association with intracellular membranes (Lee et al., 2001; Miller et al., 2001; Restrepo-Hartwig and Ahlquist, 1999). Viruses were shown to cause proliferation of host membranes possibly to amplify sites available for replication. Positive-strand RNA viruses utilize a variety of membranes as sites of replication. They range from outer membranes of endoplasmic reticulum (ER), mixed compartments of secretory pathway, endosomes, mitochondria and other organelles (Westaway et al., 1997; Pedersen et al., 1999; Suhy et al., 2000; Froshauer et al., 1988; Hatta et al., 1973). The type of membrane used is specific to the virus. In the case of BMV, modulating the levels of replication factors changed the structure of membrane rearrangement but still supported virus replication (Schwartz et al., 2004). To determine RCNMV replication sites, *Aequoria victoria* green fluorescent protein (GFP) fusions were made to both *p27* and *p88*, and GFP was used to visualize the location of these fusions in *N. benthamiana* protoplasts. Both the *p27* and *p88* proteins individually localized to the ER suggesting it as the possible RCNMV replication site (Turner et al., 2004). RCNMV infection caused invaginations of perinuclear ER and proliferation of smooth ER as further evidence of ER being replication sites.

**Trans-activation**

Capsid protein is encoded by RNA-1 and CP subgenomic RNA (sgRNA) is produced by a process of RNA-2 trans-activation. The accumulation of sgRNA corresponding to CP in vivo was very low and infrequent (Zavriev et al., 1996). The full length CP sgRNA was calculated to be 1525 bases with a 62 nucleotide 5’ leader
region that shows 13 of the 14 nucleotides identical to that of 5' RNA-1 leader. This subgenomic leader is likely to be the positive strand promoter. Sit et al., (1998) in an elegant experiment demonstrated a novel RNA-mediated regulation of transcription that resulted in the synthesis of CP sgRNA (Fig. 5). An RCNMV RNA-1 cDNA clone that expressed the GFP in place of CP was engineered. Fragments of RNA-2 were expressed from the TBSV replicon. A 34-nucleotide sequence in RNA-2 (756-789) was demonstrated to be sufficient to produce GFP as well as synthesize sgRNA corresponding to GFP. This 34-nucleotide region on RNA-2, denoted as the trans-activator (TA), is predicted to form a simple stem loop. Sequence alignments revealed that the 8-nucleotide terminal loop of this stem loop was complementary to an 8-nucleotide sequence on RNA-1, the trans-activator binding site (TABS), located within the CP subgenomic promoter. Mutations in the RNA-2 TA and RNA-1 TABS revealed that the base pairing between these two elements was a prerequisite for the trans-activation function. Based on these results a model for trans-activation in RCNMV was proposed: RNA-1 progeny molecules are synthesized in the initial phase of replication, later RNA-2 molecules are synthesized and the TA on RNA-2 base pairs with the TABS on RNA-1 thereby trans-activating CP sgRNA synthesis. This mechanism could be mediated by viral or host proteins. This base pairing could form a steric hindrance to the replicase from completing synthesis of full-length RNA-1 negative strands. The truncated complementary strands that are transcribed possibly serve as templates for the synthesis of positive-sense sgRNA. NMR and structural computer modeling were used to investigate the TA-TABS interaction at the molecular level. Synthetic oligonucleotides corresponding to both TA and TABS
were observed to form two stacked helices with the stem of RNA-2 TA stem loop forming one helix and the other formed by the intermolecular base pairing between the TA loop and RNA-1 (Guenther, et al., 2004).

Movement

Virus movement in animals takes place by means of receptor-mediated endocytosis or cell fusion. Plant viruses are unable to utilize this route due to the presence of the rigid cell wall of plant cells. A plant virus when introduced in to a plant cell replicates and then moves from the initially infected cell into neighboring cells to cause a productive infection. These viruses circumvent the rigid cell wall by moving through plasmodesmata, the intercellular connections connecting the cellular contents of plant cells (Gibbs, 1976; Atabekov and Dorokov, 1984). The viruses eventually reach the vascular transport system and then systemic spread occurs through the phloem (Gilbertson and Lucas, 1996). Virus encoded non-structural proteins termed movement proteins mediate cell-to-cell movement through plasmodesmata (reviewed in Hull, 1989; Atabekov and Taliantsky, 1990). Although there is little similarity between various virus movement proteins, comparison of viral MPs from distinct groups of plant viruses have identified conserved amino acid motifs that may reflect functional ancestry (Melcher, 1990; Koonin et al., 1991).

Infectivity studies demonstrated that RCNMV RNA-1 and RNA-2 were required for a systemic infection (Gould et al., 1981; Okuno et al., 1983) with RNA-2 required for the expansion of the local lesions (Osman et al., 1986) suggesting that the movement protein is involved in cell-to-cell movement as well as systemic
infection. In the absence of the movement protein the RNA-1 is unable to move from inoculated cells to adjoining cells although it replicates efficiently.

Deletions less than 39 amino acids in the C-terminus of the RCNMV MP exhibited wild type movement function whereas larger deletions failed to exhibit the cell-to-cell movement function (Xiong et al., 1993a). A spontaneous mutant identified from cowpea, had a single base deletion at nucleotide 790 which was responsible for a truncated MP and failed to cause a systemic infection (Osman et al., 1991). The exact mechanism of the movement function of the RCNMV MP is unknown but believed to be similar to that of tobamoviruses. The tobamovirus (TMV) movement protein accumulates in the plasmodesmata and modifies the aperture to allow passage of genomic RNA (Lucas and Wolf, 1993). The TMV MP modifies the size exclusion limit (SEL) of plasmodesmata from the 800 Da limit to more than 10 kDa in MP transgenic plants (Wolf et al., 1989). Tobamovirus MP binds to single stranded RNA \textit{in vitro}, and it is believed that the MP-RNA in the form of a ribonucleoprotein complex moves intercellularly in plants (Hull, 1989; Citovsky et al., 1992). This is further supported by the observation that TMV CP is not required for cell-to-cell movement (Culver and Dawson, 1989; Saito et al., 1990). RCNMV MP is similar to TMV as it accumulates in the cell wall (Osman and Buck, 1991), binds to single stranded RNA, increases the SEL of plasmodesmata (Osman et al., 1992; Giesman-Cookmeyer and Lommel, 1993) and does not require CP for cell-to-cell movement (Xiong et al., 1993a). The TMV movement protein has two single strand RNA-binding domains and they co-operate binding to viral RNA (Citovsky et al., 1992).
Gel shift and photochemical crosslinking studies identified a single-stranded RNA-binding domain in the region between amino acids 181 and 225 in the RCNMV MP (Osman et al., 1993). Alanine scanning mutagenesis of the RCNMV MP identified three distinct functional domains (Giesman-Cookmeyer and Lommel, 1993): an RNA-binding domain, cooperative RNA-binding domain and, a domain necessary for the cell-to-cell movement function. The RNA-binding domain resides in the N-terminal half of the MP and mutations in the amino acids 122 and 128 reduced the level of RNA binding up to 10% of wild type. However, very little RNA-binding activity is required for the cell-to-cell movement function (≤ 20% of wild type). Alanine substitutions in amino acids 27-31, 122, 128 and 305 appear to disrupt the cooperative RNA-binding domain, which is not necessary for movement. The third domain (amino acid 278) which exhibits both RNA-binding and cooperative RNA-binding is deficient in cell-to-cell movement suggesting its involvement in either targeting the MP-RNA complex to plasmodesmata, modifying the plasmodesmata in interaction with host factors or involved in proper folding of the ribonucleoprotein complex. Among dianthovirus movement proteins there is high amino acid conservation in the amino terminal region (Kendall and Lommel, 1992) and this suggests that it maybe involved in the movement function. There is very little homology between the movement proteins of RCNMV and TMV, but both have highly ordered structures with repeating hydrophobic and hydrophilic domains typical of membrane bound proteins (Xiong et al., 1993a). The function of the movement proteins is believed to be dependent more on the three-dimensional structure than on their sequence similarity (Melcher, 1990). The TMV MP was able to provide the
cell-to-cell movement function to the heterologous RCNMV and vice versa demonstrating their functional equivalence (Giesman-Cookmeyer et al., 1995b).

Transgenic *N. benthamiana* plants expressing either TMV or RCNMV MP were able to complement the cellular movement function of the heterologous RNA. Furthermore both were able to perform as helper viruses to provide cell-to-cell spread of the movement-deficient heterologous virus. The MP furthermore may be involved in host range determination. TMV upon infecting resistant plants, replicate in initially infected cells but fail to invade adjacent cells suggesting that the resistance is due to the inability of the virus to move (Sulzinski and Zaitlin, 1982).

There are many examples of plant viruses that replicate in protoplasts of non-hosts (Ponz and Bruening, 1986) and the transport function of the virus is considered to be a determinant in its host range. Although *N. tabacum* is a non-systemic host for RCNMV, RNA-1 replicates in BY-2, a cell line derived from *N. tabacum* (Ragetli and Elder, 1977; Paje-Manalo and Lommel, 1989). This suggests that the resistance observed is due to the inability of RCNMV to move in *N. tabacum* and suggests that MP functions as a host range determinant. Immunocytochemical studies on TMV movement protein have shown its localization to the plasmodesmata both in infected plants and in MP expressing transgenic plants (Tomenius et al., 1987; Atkins et al., 1991). Movement proteins of other plant viruses have also been localized to the cell wall and plasmodesmata (Albrecht et al., 1988; Godefroy-Colburn et al., 1986; Lehto et al., 1990; Linstead et al., 1988; Moser et al., 1988). The RCNMV MP was detected in the cell wall fraction of infected *N. clevelandii* leaf tissue (Osman and Buck, 1991). The sub cellular location of the RCNMV was
recently demonstrated to be the cell wall, presumably the plasmodesmata, and an intimate correlation between localization and cell-to-cell movement was identified (Tremblay et al., 2005). Similar to TMV, it has been shown that RCNMV MP was capable of modifying plasmodesmata to enable movement of macromolecules, as well as trafficking RCNMV RNA (Fujiwara et al., 1993). Although MP binds to both single stranded RNA and single stranded DNA, it was observed that it selectively transported single stranded RNA but not DNA in vivo. The RCNMV MP does not unfold the RNA in the MP-RNA nucleoprotein complex. There is further evidence of plant viral MP-GFP fusions being co-localized to the cytoskeleton (Heinlein et al., 1995; McLean et al., 1995) and ER-derived membranes (Mas and Beachy, 1999). Since these are the components of the intra- and intercellular transport machinery it is likely that the plant viruses have evolved means to subvert the intercellular communication network to facilitate the spread of infection.

Virion formation is believed to be the essential requirement for translocation of many small RNA spherical and rod-shaped viral genera such as *Tobamovirus*, *Potyvirus*, *Furovirus*, *Sobemovirus*, *Cucumovirus* and *Bromovirus* (Seron and Haenni, 1996). Long distance transport is believed to take place through the vascular system. Vascular transport is believed to occur in the form of virions in order to protect the viral genomic RNA from nuclease activity in the vasculature (Saito et al., 1990). There is also the possibility that the CP could be interacting with a host factor enabling vascular transport. TMV has been demonstrated to require CP, possibly in the form of virions for long distance transport (Saito et al., 1990). TBSV CP-deficient mutants were able to cause systemic infections in *N. clevelandii*.
and *N. benthamiana* plants, but with some delay compared to wild-type infection (Scholthof et al., 1993). However, systemic infection near wild type occurred only when the mutants were able to package into virions (Qu and Morris, 2002). TCV, a member of the *Tombusviridae* family, also requires virus assembly for vascular movement (Cohen et al., 2000; Heaton et al., 1991). RCNMV moves cell-to-cell in the absence of CP while CP is required for long distance transport depending on the host genotype and temperature. These capsid protein mutants were unable to infect either *N. benthamiana* or *N. clevelandii* systemically at 25°C although systemic spread was observed at 15°C (Xiong et al., 1993a). This has also been observed with *Brome mosaic virus* (BMV; Ding et al., 1999). Systemic infection due to cell-to-cell spread through the stem is observed 2-4 weeks post inoculation.

RCNMV RNA-1 together with RNA-2 is required for CP accumulation and virion formation, and capsid protein in the form of virions is required in the long-distance movement of RCNMV (Vaewhongs and Lommel, 1995). Although plasmodesmata SEL expanded when TMV MP was expressed in transgenic tobacco plants, it had no effect on the SEL of plasmodesmata connecting bundle sheath and phloem parenchyma cells (Ding et al., 1992). Similarly *Cowpea chlorotic mottle virus* (CCMV), when infecting a non-systemic soybean host, was not observed in companion cell-sieve element (CC-SE) although it was detected in the vascular parenchyma (Goodrick et al., 1991). Certain RCNMV movement protein mutants that facilitated cell-to-cell spread were unable to cause a systemic infection and they were confined to the bundle sheath cells and/or phloem parenchyma (Wang et al., 1998). These observations suggest that the role of cell-to-cell spread is genetically
distinct from the long distance function of the movement protein. RCNMV, when infecting \textit{N. tabacum}, a non-systemic host, causes a local infection but was restricted to bundle sheath cells and phloem parenchyma with low levels detected in the companion cells, but none detected in the sieve element. The barrier to long distance transport may reside within the phloem parenchyma-companion cell interface and plasmodesmata in this location may require both viral movement protein as well as host factors to enable movement to the vascular transport system.

\textbf{The research project}

RCNMV is a simple single-strand positive-sense RNA virus with icosahedral virions. Since infectious transcripts are available and RCNMV readily infects \textit{Nicotiana} sp. it is an ideal virus model to be studied. However, it is also a complex system where its small genome codes for all the requirements of its life cycle. Therefore exploration of the RCNMV molecular processes will likely provide novel and remarkable insights into plant virus biology.

Morphologically RCNMV is most similar to TBSV on the basis of size (34.2 nm), round virion outline and granular surface morphology (Hatta and Francki, 1984). Based on the primary amino acid sequence similarity to the TBSV capsid protein, RCNMV CP is divided into four domains (Fig. 6). The N-terminus of the CP is a flexible region composed of two domains: the RNA-binding (R) domain and the arm (a) region downstream. The arm connects the R domain to the Shell (S) domain. The R domain contains many basic amino acids and is involved in binding to the RNA and neutralizing the charged phosphates on the RNA. The globular S
domain forms the shell of the virion and it consists of two sets of four-stranded anti-
parallel $\beta$ sheet structures in a jellyroll conformation. There are clusters of negatively
charged residues with an invariant aspartic acid residue within the S domain that
may constitute a Ca$^{2+}$ binding site which maybe involved in viral assembly and/or
structural stability. The C-terminal protruding (P) domain forms surface projections
that give the virion a granular appearance (Giesman-Cookmeyer et al., 1995a). Each
projection is composed of two P domains. There is also a hinge (h) sequence that
links S and P domains, which provides CP the flexibility to change conformations.
The S and P domains of the capsid protein have a high sequence similarity with that
of Tombusvirus and a moderate sequence similarity with the Carmovirus genus
(Rochon et al., 1991).

There is very little known of RCNMV assembly and the packaging
complement within RCNMV virions is unknown. If RCNMV produces only a single
type of virions it must package both RNA molecules into a single virion. If multiple
types of virions are produced, the two RNAs could be packaged separately, or in
different combinations. Packaging of RNA-1 in one virion and three RNA-2
molecules in another virion has been suggested. This however, does not explain the
single component observed by buoyant density centrifugation of RCNMV virions in
cesium chloride. Encapsulation of both RNA segments into the same virion, as is the
case with Nodaviruses, insect-infecting bipartite RNA virus (Schneemann and
Marshall, 1998), is not possible due to the ratio of RNA-1: RNA-2 which is
approximately 1:3 (S.A. Lommel, unpublished data). *Turnip crinkle virus* which is
structurally similar to RCNMV appears to initiate assembly by forming a complex of a
trimer of CP dimers with the origin of assembly sequence on the genomic RNA. The origin of assembly of TCV was determined to be a distinct bulged hairpin loop in the CP ORF (Qu and Morris, 1997). Since RCNMV genome consists of two RNAs, each molecule may have its own packaging signal or the origin of assembly could be the secondary structure formed by the intermolecular base-pairing of RNA-1 and RNA-2.

RCNMV RNA-1 and RNA-2 of RCNMV are required for a systemic infection and CP in the form of virions is required for vascular transport. Since formation of virions is an integral part in the virus life cycle it is important to determine the processes involved in producing virions in order to characterize the infectious process of RCNMV. Formation of virions ultimately affects the virus life cycle and subsequently the disease process. Disruption of virion formation will lead to the control of the virus. With that goal in mind, this research was designed to identify the genomic signals required for assembly of virions. These signals, referred to as packaging signals or origin of assembly sequences (OAS), have been and are being studied for a number of viruses including animal viruses such as HIV-1. To date TCV is the only icosahedral plant RNA virus whose packaging signal has been completely characterized (Qu and Morris, 1997). Furthermore although virions are morphologically similar, TCV is consists of a monopartite genome whereas RCNMV contains a bipartite genome. The packaging signal of Flock house virus, an insect-infecting RNA virus with a bipartite genome, has been characterized. Although it contains two RNAs that are packaged together, the packaging signal has been identified only on RNA2 (Zhong et al., 1992; Newman and Brown, 1973; Krishna and Schneemann, 1999). In both TCV and FHV the packaging signal has been
delineated to a sequence that folds into a stem loop structure. It is important to identify the origin of assembly of RCNMV and to determine common packaging aspects between these viruses. This will provide us with insights as to the forces and mechanisms directing virus assembly. Furthermore, determining the specific packaging signal on RCNMV would enable us to utilize this knowledge in developing techniques such as viral packaging systems that are presently pursued for nanotechnology or antiviral drugs.
Fig. 1. Symptoms caused by RCNMV on *Nicotiana benthamiana*. On left, necrotic ringspot lesions (local lesions) produced on the inoculated leaf. On right, systemic symptoms include characteristic mottling, cupping and leaf distortion.
Fig. 2. The electron microscopic image of RCNMV virions. Note the spherical outline and the granular surface of the virions, a characteristic of *Tomusviridae* virus CP with protruding domains.
Fig. 3. The genome organization RCNMV. A schematic representation of RNA-1 and RNA-2. The encoded proteins and the ORFs are shown. ORF nucleotide locations of start and stop positions are indicated below. The -1 frame shift position is shown and the arrow depicts the CP subgenomic RNA promoter.
Fig. 4. The 3' termini of RCNMV RNA-1 and RNA-2. The nucleotide sequences of the 3' termini are given above and the conserved foldings of the respective 3' termini are given below.
Fig. 5. Trans-activation and CP sgRNA synthesis. RNA-1 and RNA-2 are indicated as lines. The polymerase complex is indicated as the globular structure. The TA-TABS interaction is shown.
Fig. 6. The structure of the TBSV particle architecture. RCNMV particle structure and the CP domains are similar to the TBSV. Each CP subunit folds into three domains (R,S,P) with a 35 residue connecting arm (a) between R and S and a hinge (h) between S and P. The number of amino acid residues in each structural module is indicated. The CP subunits pack into the virus particle in one of the three conformations, A (red), B (blue) and C (green). The P domains project out of the surface of the particle (from, Harrison, et al., 1978).
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Chapter 2

Virus Packaging Signals: A Review
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Introduction

Virus survival is dependent on the ability of the virus to be spread from one susceptible host to another. Plant virus spread is achieved by transmission through invertebrate hosts or mechanical means in the form of virions (Harrison et al., 1974). The primary functions of a virion are to protect its genome from degradation and to act as a transmission. Thus virion formation is an integral function of the virus life cycle.

Within the intracellular environment, plant RNA viruses disassemble and expose the viral genome to the cellular proteins for translation of viral proteins or replication of the genome. On completion of the initial life cycle stages the movement protein (MP), and/or host proteins enable the transfer of virus to neighboring cells via the plasmodesmata (Fujiwara et al., 1993). The initial virus, cell-to-cell movement, occurs through the mesophyll and the virus eventually enters the vascular system where spread throughout the plant forming a systemic infection takes place via the vasculature. Vascular movement, termed the long-distance movement occurs through the phloem for most plant viruses (Lucas and Gilbertson, 1994), although some virus long-distance movement takes place through the xylem (Gergerich and Scott, 1988). Cell-to-cell movement of most viruses e.g., Tobacco mosaic virus (TMV) takes place in the form of a nucleoprotein complex of genomic nucleic acids and movement protein (Kawakami et al., 2004). However, long-
distance movement of most viruses, including TMV, occurs in the form of virions (Dawson et al., 1988). There are a few exceptions to this. Cowpea mosaic virus requires virion formation for cell-to-cell movement and long distance movement (Wellink and van Kammen, 1989). Tomato bushy stunt virus (TBSV), on the other hand, does not require virion formation for cell-to-cell or vascular movement in Nicotiana benthamiana (Scholthof et al., 1993).

Virus structure and assembly has been the subject of various studies beginning in late 19th century. In 1956, Crick and Watson proposed a model for virus particle, where they suggested that a virus was comprised of identical capsid protein (CP) subunits arranged in a regular manner around viral RNA. Viral RNA must interact with the CP to initiate formation of this structured particle. The negative electrostatic charges on the phosphate backbone of RNA are neutralized by the basic amino acid residues in the CP and by positively charged ions (Mg²⁺, Ca²⁺, etc.). To encapsidate the cognate viral RNA genome, viral capsid protein must specifically select its genome from a pool of homologous and heterologous cellular RNAs. Viruses utilize a variety of approaches to achieve this specific packaging. A general feature of virus replication is its occurrence in association with cellular membranes (Restrepo-Hartwig and Ahlquist, 1999), which facilitates concentration of viral products into subcellular compartments. This compartmentalization excludes most other non-viral components and concentrates viral products for efficient packaging of the viral genomes.

X-ray crystallography and cryo-electron microscopic reconstructions have been used to determine the structural arrangement of a number of plant and animal
viruses (e.g., Southern bean mosaic virus - Opalka et al., 2000; TBSV- Harrison et al., 1978; Pariacoto virus-Tang et al., 2001). There is immense interest in understanding the molecular processes involved in virus assembly and recent advances such as nanotechnology for targeting drugs have greatly aided it. There are three steps involved in the formation of a stable RNA virion (reviewed in Zlotnick, 2003). First is the sequence-dependent RNA/protein interaction, which is responsible for initiation, specificity and regulation of assembly. Secondly, sequence-independent RNA/protein interactions stabilize the viral RNA packaged within the protein shell (Tellinghuisen et al., 1999; Zlotnick, 2003). Sequence-independent RNA interactions are required for the neutralization of negative charge of the RNA in order for it to be tightly packaged inside the particle. Finally protein/protein interactions stabilize the capsid shell to form the compact virion. Although capsid protein subunits possess identical surfaces they form different interactions with each other in a highly regulated manner to form the virion.

Icosahedral viruses appear to employ two distinct mechanisms for assembly: the formation of partially assembled procapsid into which the virion genome is inserted (Picornaviruses) or the formation of an initiation complex by viral RNA and capsid protein that later incorporates additional capsid protein subunits to produce an intact virion (Retroviruses). The mechanism employed by a virus however, is not clearly defined by host kingdom.

Specificity during assembly is determined by a genomic sequence/or structural element known as the origin of assembly sequence (OAS) or packaging signal that is involved in binding of viral CP to initiate the encapsidation process. In
the absence of cognate genomic RNA many capsid proteins have been shown to bind and encapsidate host nucleic acids e.g., HIV-1 (Muriaux et al., 2001) and *Cowpea chlorotic mottle virus* (CCMV). Many plant viruses such as *Turnip crinkle virus* (TCV) and *Brome mosaic virus* (BMV) preferentially package their genomic RNA over non-virion RNA (Bancroft et al., 1969; Cuillel et al., 1983; Sorger et al., 1986; Wei et al., 1990 and 1992). Similarly, HIV-1 preferentially packages full length genomic RNA in the presence of spliced RNA (Clever and Parslow, 1997). However, heteroencapsidations have also been reported, where the capsid protein of one virus packages the RNA of another and this is evidenced by *Potato leaf roll virus* (PLRV) that encapsidate *Potato spindle tuber viroid* genomes (Querci et al., 1997).

The first determination of an OAS was in the helical plant virus TMV, genus *Tobamovirus*. Infectious TMV virions can be reconstituted *in vitro* from purified viral RNA and capsid protein (Fraenkal-Conrat & Williams, 1955). This approach was utilized to delimit the minimal OAS to approximately 1000 nts from the 3' terminus of the TMV genome. Deletion analysis revealed the strict requirement for a 75-nucleotide stem loop for assembly (Turner & Butler, 1986). Mutagenesis of the loop sequence revealed the necessity for a G residue as every third nucleotide in the OAS (Turner et al., 1988). The presence of the TMV OAS was proven *in vitro* and *in vivo* by the formation of pseudovirions consisting of heterologous RNA transcripts containing the minimal OAS encapsidated by the TMV capsid protein (Sleat et al., 1988a; Sleat et al., 1988b; Sleat et al., 1986). Although the TMV OAS is a simple stem loop in the 3' region of the genome, the structure and location of the OAS can differ from viral species to species.
The OASs of a number of viruses have been vigorously studied in order to identify chemicals to disrupt the packaging process as an antiviral drug strategy. This is a promising approach for HIV-1 (Dorman and Lever, 2001) and *Herpes simplex virus* (Visalli and van Zeijl, 2003). Since there is no process in the host cell analogous to viral assembly it is thought that these chemicals should be safe.

Elucidation of virus assembly mechanisms would give us great insight into the processes governing virus infections and enhance our understanding of these economically important pathogens. The information could also be utilized to control plant viruses of agronomical importance.

### Animal virus OASs

Packaging signals/sequences have been determined for both RNA and DNA animal viruses. Encapsidation signals have been identified for a number of viruses including the members of *Coronaviridae* (Narayanan and Makino, 2001), *Togaviridae* (Weiss et al., 1994), *Bunyaviridae* (Severson et al., 2001; Xu et al., 2002), and *Retroviridae* (McBride and Panganiban, 1996; Banks and Linial, 2000). Most of these OASs are represented by a single stem loop or hairpin structure that has a high affinity for the cognate CP.

### Retroviruses (family *Retroviridae*)

Retroviruses are a large family of enveloped RNA viruses that replicate by reverse transcription and integrate into the host genome. Many are animal cancer-causing viruses. They are responsible for many human diseases including arthritis,
malignancies, immunodeficiencies, and neurological disorders. Retroviruses are the only RNA viruses to be truly diploid. A retrovirus particle contains ~ 1,500 Gag molecules arranged radially with the N termini facing outward and the C termini inward (Swanstrom and Wills, 1997). In the interior is the dimeric positive sense genomic RNA with ~30 molecules of tRNA and other small cellular RNAs (Berkowitz et al., 1996). There is also virus-encoded protease, reverse transcriptase and integrase within the particle. A lipid bilayer derived from host-cellular plasma membrane surrounds the Gag shell and virus-encoded envelope proteins extend through it. After the release from the cell the virion undergoes a maturation process in which the Gag protein is cleaved by protease into matrix (MA), capsid (CA) and nucleocapsid (NC) (Swanstrom and Wills, 1997). Maturation results in the reorganization of the virion internal structure, which is critical for infectivity of the virus.

The retrovirus genome consists of two homologous single-stranded RNAs that are base paired near their 5’ ends by a non-covalent dimer linkage (Bender and Davidson, 1976). Virus encoded reverse transcriptase transcribes the retroviral RNA genome into a double stranded DNA intermediate which is integrated into the host genome leading to the formation of the pro-virus (Temin and Mizutani, 1970). New viral genomic RNA is transcribed by the host machinery and recognized by viral proteins and encapsidated. The encapsidation signal (Ψ) resides in the same region of the genome that is spliced out of the genomic message near the dimer linkage (Fig. 5; Linial and Miller, 1990). Retroviral packaging cell lines are powerful tools for studying gene transfer because integration of the viral DNA into the host genome
results in stably inherited genes. These cell lines are defective in packaging their genomic RNA into virions but can provide in trans, the proteins necessary for virion production. They effectively complement replication-defective but packageable retroviruses (Mann et al., 1983).

The first animal virus OAS was identified in a quail cell line infected with Rous sarcoma virus (RSV), provirus mutant SE21Q1b (Linial et al., 1978). This mutant has a 179 base deletion from the 5' leader which renders it unable to package its genomic RNA, but produced virions containing host RNAs (Anderson et al., 1992; Linial et al., 1978). It was concluded that this deleted region encodes the specificity domain. Subsequently the OAS was mapped to a 160 nucleotide element between 5' untranslated region and the start of gag codon (Banks et al., 1998). The OAS sequence folds into 4 stem loops (L2, O3, L3 and L4). Stem disrupting mutations showed that the O3 was essential for encapsidation (Fig. 1). The four stem loop mutants when passaged multiple rounds in avian cells reverted to form stable stem structures while some contained nucleotide substitutions to stabilize the overall folding (Doria-Rose and Vogt, 1998). The disruption of the stem loops had deleterious effects on both replication and packaging and hence the nesting of multiple functions within a region confers a strong selection pressure on the virus.

The Avian sarcoma leukosis virus (ASLV) encapsidation sequence (AΨ) was localized to a 270 nt element containing a three-stem-loop utilizing a heterologous packaging system (Aronoff et al., 1993). Mutations in AΨ were carried out to disrupt the secondary structure to determine its significance in RNA packaging (Knight et al., 1994). The mutants were assayed for their ability to package the heterologous
lac Z reporter gene into virions when co-transfected with a non-packageable helper virus and it was observed that a base-paired stem structure was at least in part required for efficient encapsidation. The packaging signal was relatively position independent but displayed a reduced function when inserted in the opposite orientation. ASLV subgenomic RNA (sgRNA) exhibited packaging when the AΨ was included in proper orientation (Mann and Baltimore, 1985). The minimal packaging sequence of ASLV is located between nts 156-237 (Fig. 2) and it forms an O3 stem and three stem loops named O3Sla, O3SLb and O3SLc (Banks and Linial, 2000).

The interaction between Ψ and the upstream primer binding site (PBS; Fig. 5) was postulated to enable access of Gag to the packaging element. In support, RNA transcripts representing Ψ and PBS elements formed loose complexes in solution. Enzymatic probing of these complexes with RNase T1, antisense oligonucleotides and site-directed mutagenesis established that there were base pairing interactions between the internal loop of PBS and the terminal loop of O3Sla (Kanevsky et al, 2003). This five base pair interaction forms a pseudo-knot structure, which may play role in packaging as well as translation and tRNA binding.

The OAS of the Spleen necrosis virus (SNV) was delimited to 185 nucleotides within the genomic leader sequence (Watanabe and Temin, 1982) and further deletion analysis narrowed the encapsidation site to 144 nt between the long terminal repeat and the start of the gag gene (Embretson and Temin, 1987). This region is spliced out during mRNA maturation. Linker scanning and site directed mutagenesis of the encapsidation sequence revealed a double hairpin motif and while the order of the two hairpins was not critical, the GACG sequence in at least
one of the loops was significant for function (Yang and Temin, 1994). Comparison of
the 5' untranslated regions of SNV and the member virus, *Moloney murine leukemia
virus* (MoMuLV) by computer sequence alignment, RNA structural properties, and
free energy analysis revealed a significant 70-80 nucleotide double hairpin motif on
MoMuLV. The 80 nt MoMuLV double hairpin element could be substituted for the 80
nt element of SNV without a loss of packaging efficiency (Yang and Temin, 1994). A
similar encapsidation signal has been identified for *Feline leukemia virus* with a
predicted structure of two stem loops, with the GACG loop sequence, situated
between the splice donor site and the *gag* gene (Burns et al., 1996).

Although, SNV can package sequences containing the 80 nt double hairpin
element from MoMuLV, MoMuLV on the other hand did not exhibit reciprocal
packaging (Beasley and Hu, 2002). MoMuLV Gag requires additional 5' flanking
elements for optimal packaging. Chimeras containing the double hairpin element
with heterologous flanking regions from MoMuLV and SNV were created and
MoMuLV Gag rescued RNA packaging of only the chimeras containing the MoMuLV
5' flanking region. MoMuLV Ψ is a 350 nucleotide element (212-563) upstream of
the Gag start codon (Fig. 3 A; Mann et al., 1983). Similar to HIV-1, it is a structurally
organized domain consisting of 4 stem loops (DIS-1, DIS-2, SL-C and SL-D). The
linker sequence, UCUG, present in both DIS-1 and DIS-2 bound to NC protein with
high affinity (Ferbitz et al., 2004). This linker is base paired and not available for
binding to NC in the monomeric form of the genomic RNA. However, upon
dimerizing (which could involve either DIS-1 or DIS-2 or both) the UCUG linker
sequence becomes exposed for NC binding enabling encapsidation (Fig. 3 B). The
CCHC (where C=cysteine and H=histidine) zinc knuckle of NC binds to the UCUG linker. NMR chemical shifts and nuclear Overhauser effect (NOE) cross peak patterns were used to determine the structural interaction between the zinc knuckle domain of NC and the Ψ sequence. MoMuLV NC is partially formed in solution and folds to form the binding pocket only in the presence of Ψ signal. Mutants that had stabilized intramolecular base-pairings did not bind to NC while the mutants with stabilized base pairings in the dimeric form bound to NC with high affinity. Thus it was demonstrated that the Ψ signal of MoMuLV acts as a conformational RNA switch that functions to ensure encapsidation of only the diploid genome (D'Souza and Summers, 2004). MoMuLV Gag packaged cellular RNA in the presence of packaging signal-deficient genomic RNA. However the produced virions were unstable suggesting that the genomic RNA in MoMuLV not only is involved in nucleating RNA-Gag initiating complex but possibly maintains the integrity of the virion (Muriaux et al., 2001).

**Human immunodeficiency virus type -1**

HIV-1, genus *Lentivirus*, is the causal agent of acquired immune deficiency syndrome (AIDS). The virus primarily infects CD4+ macrophages and T lymphocytes resulting in a drastic reduction in the number of these associated with full immune deficiency. A dimer of HIV-1 genomic RNA is specifically incorporated into an assembling virion. The RNA encapsidation of HIV-1 involves the recognition of an encapsidation signal in the genomic viral RNA by the viral Gag polyprotein. Structural and genetic analyses have shown that this event involves the interaction
between a highly structured region of the 5’ end of the viral genomic RNA and amino acids in the nucleocapsid domain of the Gag protein. The recognition of cis-elements (Ψ), within the genomic RNA 5’ leader by the Gag polyprotein leads to virion formation. The specificity of packaging is believed to depend largely on direct binding interactions between the viral polyprotein Gag- the main structural component of HIV-1 capsids- and the packaging signal. The HIV-1 Ψ region is a cluster of four stem loops designated SL1 to SL4 (nts 243-352) (Fig. 4B; McBride & Panganiban, 1997). Although the SL3 stem-loop has been shown to direct encapsidation by itself, the first 400 bases of the genome were also implicated in assembly and this includes not only SL1 to SL4 but also 242 bases upstream (Helga-Maria et al., 1999; Kaye et al., 1995; McBride et al., 1997).

This recognition event is important for the exclusive encapsidation of genomic length viral RNA, in the presence of spliced viral RNAs and cellular mRNAs. In most retroviruses the encapsidation signal is located between the major subgenomic splice donor site and the start of the gag codon and therefore the spliced message is excluded from packaging. In HIV-1 the primary encapsidation signal is located downstream of the primer binding site and extends into the gag gene overlapping the major splice donor (Fig. 5; McBride & Panganiban, 1997). The stem loops 1, 3 & 4 have been shown to contribute to the encapsidation of HIV-1, whereas SL2 (major splice donor site) does not appear to be a critical factor (Harrison et al, 1998; Luban and Goff, 1994; McBride & Panganiban, 1996, 1997). SL3 is known to specifically interact with the NC domain for assembly. Two zinc finger motifs in the NC domain are known to form hydrogen bonds with the exposed guanosines on the SL3 loop.
(Fig. 4A) providing the main interaction between Gag and $\Psi$ (De Guzman et al., 1998). Recent data indicate that the conformation of SL3 is altered upon Gag protein binding and that the purine-rich internal loop in SL3 provides further contacts for Gag protein (Zeffman et al., 2000). SL1, although present in both genomic-length viral RNA and spliced viral RNA, seems to act in conjunction with SL3 and SL4 (McBride & Panganiban, 1997). Sequences that can influence encapsidation have also been identified upstream of the encapsidation signal (McBride et al., 1997) and in the $\text{env}$ gene (Kaye et al., 1995).

Mutations altering the base pairing at stems of SL1, SL3 and SL4 resulted in a threefold reduction in virion formation (McBride & Panganiban, 1997). Second site mutations that restored base pairing of the stem loops did not exhibit wild-type levels of encapsidation. This indicates that the structure as well as the sequence of the RNA elements contributes to the specificity of HIV-1 encapsidation (McBride & Panganiban, 1997). Although SL3 alone is sufficient to direct packaging, deletions of SL1 and/or SL3 resulted in five- to ten fold reductions in encapsidation indicating that there could be secondary elements involved in encapsidation although to a lesser degree than SL1 and SL3. The requirement of SL1 and SL3 is also position-dependent. In mutants where the positions of the two stem loops were interchanged the encapsidation levels were comparable to when one of the stem loops was deleted (McBride & Panganiban, 1997). These data point to the fact that SL1, SL3 and SL4 forms a higher-order structure that is responsible for the specificity in the interaction with Gag.
SL3 is of particular interest because the sequence is highly conserved among different HIV-1 strains. The GGNG motif on SL3 is a commonly occurring motif in the 5' leader of primate lentiviruses. Biochemical analysis revealed that both the internal loop and the tetraloop in SL3 are primary sites for interaction with Gag polyprotein. The basic residues, arginine to lysine from positions of 3 to 10 of NC, form a $^{310}$ helix that interacts with the RNA major groove (De Guzman et al., 1998). The two NC zinc knuckle motifs designated as F1 and F2, form hydrogen bonds with the exposed guanosines on the SL3 loop (Fig. 4 A&B). The remaining tetraloop bases project away from the stem and interact directly with the NC protein. Both zinc-binding domains bind specifically to the exposed guanosines forming hydrogen bonds that would normally form Watson-Crick base pairing and this may serve as a primary mode by which the CCHC zinc-binding domain interacts, sequence specifically with RNA.

The Gag polyprotein has been suggested to bind to the SL3 stem-loop and then exploit the flexibility of the internal loop to initiate unwinding of the structure with consecutive multimerization of the Gag molecules (Zeffman et al., 2000). The unwinding of the RNA would begin at the poorly structured purine-rich internal loop progressing throughout the viral RNA ultimately ending with the Gag protein coating the entire RNA.

Dimerization of two homologous strands of genomic RNA is an essential feature of retroviral replication and the conserved stem-loop sequence spanning SL2, known as the dimerization initiation site (DIS), is primarily responsible for this process. The DIS loop contains a hexanucleotide palindrome flanked by highly
conserved 5’ and 3’ purines and the DIS base pairs through a kissing loop-loop interaction with a similar sequence motif in the exposed loop of the DIS hairpin (Paillart et al., 1996; Rist & Marino, 2002). The HIV-1 5’ leader has two alternate conformations that differ in the presentation of the DIS hairpin (Fig. 5). The branched multiple-hairpin structure (BMH) conformation folds the DIS hairpin whereas in the long distance interaction (LDI) conformation, the DIS is base paired and therefore is unavailable to initiate dimerization.

Thermodynamically LDI is the most stable conformation. Wild-type HIV-1 RNA is predominantly in the LDI conformation and less than 10% is in the BMH form (De Smit and van Duin, 1990). This LDI: BMH equilibrium is the ideal for efficient packaging of RNA and a concentration shift towards either LDI or BMH negatively affects both RNA dimerization and packaging indicating that both forms and the ratio are required for dimerization and packaging of HIV-1 (Ooms et al., 2004). The region encompassing DIS and Ψ acts as a riboswitch that switches the conformation from LDI to BMH at latter stages to enable dimerization and consequently packaging of the genomic RNA. RNA dimerization has been suggested to be coupled to RNA encapsidation and this would be an efficient system to ensure that only the dimeric genome is packaged (Fu et al., 1994; Sakuragi et al., 2003). The fact that RNA signals important for both these functions overlap in the HIV-1 genome explains their intimate relationship. Fu & Rein (1993) suggested that a less stable dimer is encapsidated and that it forms a stable duplex dimer upon virus maturation. In vitro studies have shown that the NC domain induces the homodimers to form stable dimers, demonstrating the functional importance of the nucleocapsid protein as a
nucleic acid chaperone (Muriaux et al., 1996). However, it is also possible that host factors may play a role in genome dimerization.

The Bovine leukemia virus (BLV) encapsidation signal was mapped by deletion analysis to two regions in the genome. The signal was discontinuous with the first sequence situated downstream of the primer binding site and the second, a 132-nucleotide sequence within Gag gene (Fig. 6; Mansky et al., 1995). Further analysis established the primary encapsidation signal as the region located just downstream of Gag start codon which consists of two stable stem loops, SL1 and SL2 (Mansky and Wisniewski, 1998). The stem loops also has replication functions. Nucleotide base substitutions and base-switching to maintain the stem structure of these stem loops indicated that the primary sequence contributes to the function of the signal (Mansky and Gajary, 2002). The order of the stem loops was critical and the stem loops were not functionally equivalent.

HIV-1 OAS is the best studied packaging signal and has been identified down to the molecular level. Although other viral OASs may not be identical, this information can be instrumental in relating its packaging function to other viruses with unknown packaging mechanisms.

Alphaviruses (family Togaviridae)

Many alphaviruses are human pathogens. The members are enveloped with a single-stranded positive-sense RNA genome. The envelope is a lipid bilayer membrane that is derived from the infected host cell. Alphaviruses synthesize large amounts of both genomic and sub genomic RNA, yet only the genomic RNA is
packaged. This implies the existence of the OAS on genomic RNA which is not copied as the subgenomic RNA. These viruses also generate many defective interfering RNAs (DI RNA). DI RNAs are formed with serial passage of the virus at a high multiplicity of infection. They are deletion mutants of the parental virus and require coinfection with the parent virus for replication. The deleted genomes may lose all their protein coding functions, but retain replication and packaging signals. Comparison of the parent virus genome to the DI genome is useful in identifying these signals. Sindbis virus the prototype member of the Alphavirus genus has an 11.7 kb genome with a poly (A) tail. The nucleation site for the Sindbis virus was determined to be 572 nucleotides in length located 683 bases from the 5' terminus and within the nsP1 gene (Weiss et al., 1989). This sequence also played a role in the encapsidation of DI RNAs. Capsid binding activity was further delimited to a 132 nucleotide element (nts 945 to 1076) and it bound specifically to a 68-amino acid peptide derived from the capsid protein. A replicon of Sindbis virus was synthesized, that lacked the structural protein genes, but contained a heterologous sequence (lac Z gene) under the control of its subgenomic promoter (Bredenbeek et al., 1993). When the 132-mer was expressed from its subgenomic promoter, instead of the lac Z gene, a significant increase in packaging of the subgenomic RNA was observed (Frolova et al., 1997). The Sindbis virus replicon packaging helper system was used to identify the packaging signal of Ross river virus (RRV), another alphavirus. Fragments of the RRV genome were inserted into the Sindbis virus replicon downstream of its subgenomic promoter, replacing lac Z gene. When a defective helper virus provides RRV structural proteins, some of the fragments of RRV
genome were packaged and the packaging was most effective when sequences in the *nsP2* gene were used. Furthermore, the sequence from 2902 to 3062 exhibited the highest packaging efficiency and these facts demonstrate that the packaging signal of RRV is distinct from *Sindbis virus* whose packaging signal resides within the *nsP1* gene.

*Semliki forest virus* (SFV), an alphavirus, has a 13 kb genome with a poly (A) tail. DI RNAs of this virus are packaged less efficiently than the genomic RNA although they contain multiple copies of the packaging signal (Kääriäinen et al., 1981). Three DI RNAs were examined by electron microscopic heteroduplex analysis of complementary DNA (Alanen et al., 1987) and they had 90 nucleotides in common near the 2740 nt position. This data and two other DI RNAs that were sequenced suggest that the SFV OAS is situated between nts 2737-2993.

*Mouse hepatitis virus* (family *Coronaviridae*)

*Mouse hepatitis virus* (MHV) is the type species of the *Coronavirus* genus in the family *Coronaviridae*. MHV consists of a positive sense, single-stranded RNA genome and 31 kb in length. The viral RNA binds to a 50 kDa nucleocapsid that forms a helix contained within an envelope (Sturman et al., 1980). MHV produces three types of DI RNAs with different replication and packaging abilities. The type 1 DI is nearly of genomic length and can replicate and package without the help of the parental virus (Makino et al., 1988). Type 2 requires the help of the parental virus for replication and is not efficiently packaged into MHV virions (Makino et al, 1985 and 1988). Type 3 DI RNAs require a helper virus for replication but are efficiently
packaged (Makino et al., 1990). A comparison of sequences between a Type 2 DI that did not package and a Type 3 DI that did package narrowed the OAS to a region of 1480 nts which encompassed three different domains of the parental virus genome (Makino et al., 1990). When these 1480 nts were inserted into the Type 2 DI, the hybrid was efficiently packaged, confirming that this region contained the encapsidation signal. The encapsidation signal was further delimited to a 650 nt fragment at the 3’ end of the polymerase gene of the parental virus by deletion mutagenesis (van der Most et al., 1991) and to a 190 nt element by deletion analysis of the Type 3 packaging competent DI. Computer based modeling predicted this region to contain a main stem loop with a smaller 69 nucleotide stem loop projecting from the side. Further mutational studies revealed that this 69 nucleotide loop was important in retaining packaging ability and the mutants with altered sequence that did not result in altered structure were efficiently packaged (Fosmire et al., 1992). Further deletions delimited the minimal OAS to be 61 nucleotides (Fig. 7) between 20,356-20,416 nt from the 5' end of the MHV genomic RNA (Fosmire et al., 1992).

In infected cells MHV produces genomic RNA (mRNA 1) and six to eight species of subgenomic mRNAs (Lai et al., 1981; Leibowitz et al., 1981). The 5’ leader and the 3’ terminus are shared among these viral mRNAs. Only the genomic RNA is packaged into virions whereas the subgenomic mRNAs are not incorporated (Makino et al., 1990). MHV produces three envelope proteins, M, S and E. The proteins M and E are essential for envelope formation (Kim et al., 1997). Viral genomic RNA is bound to the N protein to form a nucleocapsids core (Escors et al., 2001). Narayanan and Makino (2001) demonstrated that the M protein bound only
to genomic mRNA containing the packaging signal and that this nucleoprotein
complex was selectively packaged. This is the first example of viral envelope protein
and RNA packaging signal specifying selectivity for packaging.

**Hepadnaviruses (family Hepadnaviridae)**

Hepadnaviruses are small DNA viruses that cause liver infections leading to
cirrhosis and hepatocellular carcinoma. Members of the family have encapsidated
genomic DNA but replicate by reverse transcription. There are two groups in the
family; the mammalian group designated by the type virus, *Human hepatitis B virus*
(HBV), and the avian group represented by *Duck hepatitis B virus* (DHBV) and
*Heron hepatitis B virus* (HHBV). HBV produces isometric virions of 42-47 nm in
diameter with a lipid membrane and has a 3.2 kb double stranded relaxed circular
DNA genome (reviewed in Ganem and Schneider, 2001; Seeger and Mason, 2000).
After infection of a hepatocyte, the genomic DNA is translocated to the nucleus
where cellular RNA polymerase transcribes it to produce pregenomic RNA (pgRNA).
In the cytoplasm, the pgRNA is translated to produce viral-coded reverse
transcriptase (P) and core proteins (C). Pre genomic RNA is encapsidated along
with reverse transcriptase and genomic DNA is transcribed within capsids and
mature virions containing viral DNA buds into ER and secreted out of cells. The
encapsidation signal of hepadnaviruses (ε) is located in the 5’ end of the pgRNA and
this has been shown to fold into a conserved secondary structure (Pollack and
Ganem, 1993). In HBV the 60 nucleotide ε (Fig. 8) is predicted to form a bulged
stem loop.
NMR studies to determine structure of this bulged stem loop showed that instead of the 6-nucleotide loop the apical stem is capped by a UGU tri-loop, C-G base pair and a bulged out C (Flodell et al., 2002). This fold was demonstrated to be essential for the binding of reverse transcriptase. The P protein was demonstrated to bind to the encapsidation signal on pgRNA and required for encapsidation. The ε also functions as a replication signal and the bulged region of the stem loop is utilized as the origin for minus-strand DNA synthesis (Nassal and Rieger, 1996). Reverse transcriptase binds to the apical stem loop and synthesizes a 4-nucleotide DNA primer using the bulge as a template and the primer is then translocated to the 3' end of the pgRNA for DNA synthesis (Ganem et al., 1994). The encapsidation signals of both DHBV and HHBV are similar to HBV and they too consist of a lower stem, a bulge and an upper stem followed by an apical loop (Fig. 9; Hu et al., 2004). Both share a conserved apical loop sequence. The HBV ε sequence when expressed fused to the heterologous sequence lac Z packaged into virions that contained the heterologous sequence (Pollack and Ganem, 1993). However, the encapsidation signal of DHBV appeared to be more complex in nature with a second cis-acting encapsidation element mapped to a 200 nucleotide region downstream of ε (Calvert and Summers, 1994).

**Adenoviruses (family Adenoviridae)**

Adenoviruses are non-enveloped icosahedral virions with double-stranded DNA genomes. The spherical particles are 70-90 nm in diameter. A "penton fiber" (a slender shaft with a globular head) projects from the 12 apices of the virus.
Adenoviruses are usually mild pathogens, and can cause respiratory illness or conjunctivitis. Adenovirus assembly has been of interest due to their use as gene therapy vectors (Hitt et al., 1997). Assembly of adenovirus particles is a multistep process. Viral DNA is selectively packaged into preformed capsids late in the life cycle (D'Halluin, 1995). The assembly of adenoviruses takes place via specific recognition of a cis-acting motif in the genome by viral and/or cellular proteins (Hammarskjold and Winberg, 1980; Hearing et al., 1987). The packaging domain of *Human adenovirus* (HAdV) is located at the left end of the viral genome between nts 194 and 380 (Hearing et al., 1987; Grable and Hearing, 1990, 1992; Schmid and Hearing, 1997). This consists of seven functionally redundant A repeats I to VII. As the name denotes, the repeats are AT rich and follow a hierarchy of importance with AⅠ, AⅡ, AⅤ and AⅥ being the most dominant. The repeats exhibit a bipartite consensus motif, 5'-TTTGN8CG-3' which is conserved across a number of HAdV serotypes. The packaging domain is position dependent and must be situated within the 600 bases of the 5' terminus of the genome for its proper function (Hearing et al., 1987). The packaging domains of *Bovine adenovirus* (Xing et al., 2003) and *Porcine adenovirus* (Xing and Tikoo, 2004) follow a similar pattern to HAdV, however, some of the repeats of *Porcine adenovirus* contain a tripartite motif with A/T sequences flanked by G/C-rich sequences.

*Flock house virus* (*family Nodaviridae*)

The nodaviruses are non-enveloped insect viruses with a bipartite, positive sense RNA genome packaged within an icosahedral virion. *Flock house virus* (FHV)
genome consists of two RNA molecules; RNA1 is approximately 3.1 kb and encodes the viral replication proteins, and RNA2 is 1.4 kb and encodes the viral coat protein. FHV shares many similarities with RCNMV. FHV is a small RNA virus with a small bipartite genome with the two RNAs similar in size to the RCNMV genome. Relating the findings on FHV OAS to RCNMV is imperative due to this similarity. FHV genomic RNAs are packaged into a single virion (Newman and Brown, 1973, Newman and Brown, 1977; Selling and Rueckert, 1984; Krishna and Schneemann, 1999). Defective interfering RNAs packaged within virions were found upon multiple passage of infection in *Drosophila* cells and these were used for identification of the OAS. The OAS was determined to be between nts 186 and 217 of FHV RNA2 and predicted to form a bulged hairpin structure (Fig. 10; Zhong et al., 1992). RNA folding predicted the packaging signal to be a stem loop with a 5-base loop and a 13-base-pair bulged stem. Deletion studies demonstrated that the N-terminus of the capsid protein was required for packaging of viral RNA and that in its absence the capsid protein loses its specificity and packages random RNAs (Schneemann et al., 1993; Dong et al., 1998). FHV capsid protein appears to contain two RNA-binding domains for recognition and binding of the respective RNAs although the mechanism remains unknown (Marshall and Schneemann, 2001).

**Plant Virus OASs**

*Tobacco mosaic virus*

TMV, a tobamovirus, is the most extensively studied plant virus due to its high titer, ease of experimental manipulation and extreme stability of the virion. TMV is an
ideal model for rod-shaped plant virus assembly systems. TMV is rod shaped, composed of a positive sense, single-stranded RNA of 6.4 kb in length (Goelet et al., 1982). TMV particles measure 300 nm X 14 nm. A single virion is composed of 2130 capsid protein sub units. The virus encodes four proteins; two RNA-dependent replicase proteins are directly translated from TMV RNA, capsid protein and movement protein are translated from subgenomic RNA. Purified TMV capsid protein when combined with purified TMV nucleic acid assembles to form infectious, rod-like virions in vitro (Fraenkel-Conrat and Williams, 1955). This led to the earliest suggestion of the existence of an origin of assembly sequence by Caspar (1956): "TMV RNA may contain the information that is necessary for it to interact with its own coat protein as well as code for the sequence of this protein". In vitro RNA protection experiments demonstrated a unique nucleation region for TMV assembly (Zimmern and Butler, 1977). When purified TMV RNA was mixed with a minimum amount of TMV coat protein, a unique site of around 300 bases became resistant to nuclease digestion. This minimal protected sequence was identified as the TMV OAS. The OAS sequence was determined and located to the region between 5,443 and 5,518 nucleotides from the 5’ end (Jonard et al., 1977; Zimmern, 1977; Zimmern and Wilson, 1976). The complete nucleotide sequence of TMV became available (Goelet, 1982) and an extended secondary structure consisting of three symmetrically spaced stem loops was proposed for the OAS (Zimmern, 1983). Through deletion mutagenesis, it was established that the 3’ proximal stem loop of 51 bases was necessary and sufficient for the initiation of viral assembly (Fig. 11; Turner and Butler, 1986). Recombinant heterologous RNA conjoined with the
extended nucleation site of TMV could be fully encapsidated into rod-like pseudovirions (Sleat et al., 1986). The sequence 5’-AAGAAGUCG-3’ with repeat of a guanidine residue in every third position of the 9 base bulged sequence at the apex of the stem loop is critical for RNA-protein recognition in the initiation of assembly (Turner et al., 1988). However the presence of G repeat triplet alone was not sufficient for assembly as was shown when the rest of the sequence was substituted with C residues. Furthermore altering the RNA folding close to the loop as well as shortening the stem had deleterious effects on assembly. Stability of the stem of a hairpin loop is important for the rate of initiation of assembly.

The OAS for the common strain of TMV is located 800-1000 nucleotides from the 3’ end of the genome within the movement protein ORF. The OASs of cowpea and tomato strains of TMV have been located to 300-500 nts from the 3’ end of the genome within the CP ORF (Fukada et al., 1980). Thus the different strains of TMV are grouped by the location of their assembly origin (Fukada et al., 1981). The OAS of the watermelon strain of TMV has been identified at a site similar to cowpea and tomato strains of TMV (Meshi et al., 1983). The secondary structure of all OASs is a single stem loop although the location and sequences vary among the tobamoviruses.

Capsid protein subunits aggregate to form a 34-subunit disk that sediments at 20 S (Diaz-Avalos and Caspar, 1998). The subunits are initially believed to form a two layer disk that immediately transitions to an imperfect helix (Butler, 1999). Once the OAS comes into contact with the two-layer helix, it is predicted that the OAS stem loop is inserted into the hole of the short helix. RNA binding reorganizes the
nucleoprotein complex into a virion-like helix and virion elongation proceeds bidirectionally (Schuster et al., 1980). Assembly in the 5’ direction occurs through the addition of short helices (Schuster et al., 1980) and the elongation in the 3’ direction is believed to involve CP (Lomonossoff and Butler, 1980) and bidirectional growth of the rod proceeds until the genomic RNA is totally encapsidated.

**Papaya mosaic virus** (family **Potexviridae**)

*Papaya mosaic virus* (PapMV) is a single-stranded positive sense RNA virus belonging to family *Potexviridae* that forms flexuous rods. Deletion analysis of the 5’ terminus located the nucleation site to be within the first 47 nucleotides. This region was characterized to be very rich in adenosine and cytosine residues, but without a definite secondary structure. Due to the lack of specific secondary structure, it was suggested that the recognition by the capsid protein was sequence specific (Sit et al., 1994). The presence of packaging signal(s) in the 5’ terminus is not a general feature of potexviruses. The *Bamboo mosaic virus*, a member of family *Potexviridae*, packages both genomic and co-terminal sub genomic RNA suggesting that the origin of assembly resides in the 3’-terminal 1000 nucleotides (Lee et al., 1998).

**Tobacco vein mottling virus** (family **Potyviridae**)

*Tobacco vein mottling virus* (TVMV) is a potyvirus, with flexuous rods (765 nm X 13 nm). Potyviruses are helical viruses with a single positive-strand RNA molecule (9.2 kb) encapsidated by ~ 2000 capsid protein subunits. Incubation of capsid protein with purified RNA produced virus-like particles that were however, shorter
than native virions (McDonald and Bancroft, 1977). Experiments carried out to determine the nuclease-resistant regions of the viral RNA established that the 5’ terminus of the RNA was protected and probably involved in initiating assembly (Wu and Shaw, 1998).

**Peanut clump virus**

*Peanut clump virus* (PCV), a member of the genus *Pecluvirus*, is a positive-sense single-stranded RNA virus. PCV genome consists of two RNA molecules packaged into two forms of rod shaped virions. The bimodal length distribution of virions is 190 nm and 245 nm (Fritsch and Dollet, 2000). The two RNAs share high sequence homology in the 3’-terminal region (Manohar et al., 1993). RNA-1 (5.9 kb) encodes two replicase-related proteins and P15, a suppressor of post-transcriptional gene silencing (Dunoyer et al., 2002a). RNA-2 (4.5 kb) encodes the viral capsid protein, a putative vector transmission protein, and the triple gene block (TGB) movement proteins (Manohar et al., 1993). Deletion mapping established the OAS of RNA-1 to be within the P15 coding region (5220 to 5513) (Hemmer et al., 2003). Since this element did not have any appreciable number of repeats of residues, it was hypothesized that the capsid protein interaction probably involves a specific RNA secondary structure. Both RNA-2 and its subgenome were encapsidated. There were two elements on RNA-2 that appeared to play a role in assembly. The major OAS resides in the 3’ proximal region (between 3370 and 4142) and a secondary OAS within the capsid protein coding region (391 to 1011). Although PCV contains two OASs, the secondary OAS is believed to be kinetically disfavored.
**Turnip crinkle virus (family Tombusviridae)**

*Turnip crinkle virus* (TCV) is a member of the *Carmovirus* genus in the family *Tombusviridae*. Unlike the other plant viruses described previously, TCV is an icosahedral virus (35 nm T=3 virions) with a positive sense, single-stranded RNA of ~4 kb. RCNMV, a related member, may share similarities in assembly functions with that of TCV. TCV has a monopartite genome and therefore its packaging may be conceptually less complex than RCNMV with a bipartite genome. Under high pH and ionic strength, TCV will dissociate to produce free capsid protein and a ribonucleoprotein complex of viral RNA bound to six capsid protein subunits. This ribonucleoprotein complex is thought to be the initiating structure for TCV assembly (Sorger et al., 1986). This ribonucleoprotein complex was purified by chromatography and treated with RNase to isolate the genomic RNA fragments that were bound by capsid protein (Wei et al., 1990). There were two possible candidates for the OAS, one in the polymerase open reading frame and the other in the capsid protein-coding region. The fragments were sequenced and were predicted to form highly ordered structures. Mutations in the putative candidate in the polymerase gene affected replication and not encapsidation thereby ruling out its function as the assembly nucleation site (Wei et al., 1992). Northern blot analysis of TCV capsid protein defective mutants revealed that the mutants were unable to assemble into virions when capsid protein subunits were supplied in *trans* by a TCV helper virus. This suggested that the OAS was most likely within the capsid protein open reading frame. Deletion mutations through the CP-coding region were used to identify and characterize the OAS (Qu and Morris, 1997). The mutants were constructed with a
125 nt marker sequence from rice small nuclear RNA (snRNA) and capsid protein was supplied from a TCV helper virus in a mixed infection. Packaging efficiency of the mutant TCV genome was detected with a rice snRNA specific probe. The putative OAS was located to a 186 nucleotide sequence within nucleotides 3,606 to 3,792 from the 5' end. This location of the TCV OAS was confirmed by demonstrating that a chimeric virus in which the capsid protein gene of TBSV was replaced by the TCV capsid protein gene could package heterologous TBSV RNA. Further mutations in this region narrowed the essential OAS element to a 28 nt sequence spanning nucleotides 3,765-3,792 (Fig. 12). However, adjacent sequences were also observed to be necessary for optimal assembly. This 28 nt essential OAS element was predicted to fold into a bulged hairpin loop structure.

*Turnip yellow mosaic virus*

*Turnip yellow mosaic virus* (TYMV), genus *Tymovirus*, is a non-enveloped small icosahedral virus (28 nm T=3 virions) with a positive-sense single-stranded RNA genome. Genomic RNA (6.3 kb) is 5' capped with a tRNA-like folding at its 3' end. The 5' leader is 90 nts in length and by means of structure probing and sequence comparisons it was demonstrated to fold into two hairpins with internal loops formed by C-C and C-A mismatches (Fig. 13; Hellendoorn et al., 1996). NMR and UV melting experiments have shown that under slightly acidic conditions (pH 5) the cytosines get protonated and form base pairs thereby stabilizing the stem loop. A successful infection was dependent on these protonatable cytosines (Hellendoorn et al., 1997). The proximal hairpins are proposed to be in an initial destabilized state
that enables translation. TYMV assembly takes place in chloroplasts and active photosynthesis generates slightly acidic conditions whereby the hairpins get protonated and stabilized. These protonated and stabilized hairpins function as encapsidation signals (Bink et al., 2002; Bink et al., 2003). This is another example where an initially inactive element switches its structure to become a functional OAS. Furthermore these protonatable hairpins make up a novel type of RNA-protein interaction whereby the capsid protein binds to protonated cytosines.

**Cowpea chlorotic mottle virus (family Bromoviridae)**

*Cowpea chlorotic mottle virus* (CCMV), a member of the *Bromoviridae* family, is a small positive-strand RNA virus with 28 nm icosahedral particles. CCMV produces three types of virions-which are required for infectivity- encapsidating four single-stranded RNAs. RNA1 and RNA2 encode RNA-dependent replication proteins and are packaged into separate virions. RNA3 encoding viral movement protein and RNA4, a subgenomic RNA transcribed from RNA3 encoding capsid protein, are co-packaged into a third virion. *In vitro* assembly of a spherical virus from purified RNA and capsid proteins was first demonstrated using CCMV by Bancroft and Hiebert (Bancroft and Hiebert, 1967; Bancroft et al., 1968). Conditions of low pH (<5.5) and moderate ionic strength (\(i=0.2-1.0\)) caused capsid proteins to self-assemble into empty particles which are not observed *in vivo* (Bancroft et al., 1968). The structures of empty virions and RNA-containing virions were morphologically similar. Light scattering analysis of *in vitro* assembly of capsid protein sub units indicated that the capsid protein is regularly in the form of a dimer
and that the dimeric capsid protein forms a pentamer of dimers (Zlotnick et al., 2000). The pentamer of dimers bind to viral RNA and initiate assembly. C-termini of two CP subunits form non-covalent interactions to form a capsid protein dimer (Zhao et al., 1995). The N-terminus with a high proportion of basic residues is required for in vitro assembly of RNA-containing virions but not for empty pseudovirions. Titration of RNA by capsid protein and gel electrophoresis on a native agarose gel demonstrated three specific steps in assembly. The first step is the fast pathway in which the capsid protein dimer binds to the specific viral RNA with high affinity capsid protein binding sites. This is followed by the slow pathway in which the capsid protein dimers and RNA fold to form a nucleoprotein complex. Finally the capsid protein dimers bind to the nucleoprotein with high co-operativity to form a virion (Johnson et al., 2004). CCMV in vitro assembly of empty virions are extensively characterized and these protein cages have great potential to be utilized as constrained reaction vessels for material synthesis and/or entrapment in the field of nanotechnology (Douglas et al., 2002; Douglas & Young, 1998, 1999).

**Brome mosaic virus (family Bromoviridae)**

*Brome mosaic virus* (BMV), like CCMV, is a tripartite positive-strand RNA of which the OAS has been studied extensively. The protein specificity was determined to reside in the capsid protein N-terminus that consists of an arginine-rich motif (ARM; Choi et al., 2000). Deletions of selected arginine residues in this ARM region resulted in a loss of specificity for packaging viral RNA. In the case of RNA determinants for encapsidation, multiple regions in genomic RNA appear to confer
specificity to capsid protein binding (Duggal and Hall, 1993). The BMV genomic RNA 3’ terminus folds into a tRNA-like structure (TLS) and it appears to be involved in assembly. In mutants lacking the TLS, addition of heterologous tRNAs appeared to mediate assembly (Choi et al., 2002). When RNA3 packaging elements were studied, it was observed that it contained a bipartite OAS (Choi and Rao, 2003). Both the 3’ terminal TLS and a 187 nucleotide element (nts 601-817) in the movement protein open reading frame that folded into two branched stem loops were required for assembly. The TLS was hypothesized to act as the nucleating element involved in forming the initial complex with the capsid protein pentamers, which then would facilitate the specific interaction with the 187 nucleotide primary OAS element leading to virion formation. Concurrently another lab proposed that the cis-acting element involved in packaging was the 69 nucleotide element (nts 866-934) encompassing two stem loops in the RNA3 movement protein ORF 3’ proximal sequence together with the TLS (Damayanti et al., 2003).

Summary

Viral genomes are specifically packaged by encapsidating proteins in a mix of heterologous RNAs. This is achieved by the unique packaging signals/sequences (OAS) within viral nucleic acids that are exclusively recognized by the cognate capsid protein followed by subsequent virion formation. This specific recognition of OAS is a critical step in a virus life cycle that ensures the exclusive packaging of its genome. A simple or a complex stem loop functions as an OAS for many RNA viruses. Conversely, DNA viruses utilize specific sequences and/or modifications to
sequences as packaging signals. In RNA viruses the secondary structure has a functional significance in assembly although the nucleotide sequence identity also plays a role. Although the essential OAS is defined as a simple stem loop, the sequences flanking this are also required for optimal packaging for many viruses. The OASs are generally discrete, with the exception of BLV, which has a discontinuous signal. The MoMuLV and HIV-1 viruses expose their OASs to the encapsidating proteins at the latter stages of the virus life cycle to ensure that all conditions required for assembly are met. This is achieved by the RNA acting as a riboswitch that changes its three dimensional structure in a very unique manner in order to regulate its function. Assembly function also overlaps with other critical functions such as replication, dimerization etc. Nesting of such functionally critical sequences may have evolutionary advantages to the virus.
Fig. 1. Predicted secondary structure for Rous sarcoma virus encapsidation (Ψ) signal (Doria-Rose and Vogt, 1998)
Fig. 2. The secondary structure model of the *Avian sarcoma leukosis virus* packaging region. 5’ and 3’ ends are indicated (Banks and Linial, 2000).
Fig. 3. Secondary structure of the *Moloney murine leukemia virus* encapsidation signal (Ψ). A. The core encapsidation signal of *Moloney murine leukemia virus*. B. DIS-2 forms an alternate conformation upon dimerization that exposes the UAUCUG element (in red) (from D’Souza and Summers, 2004).
Fig. 4. HIV-1 packaging signal (Ψ). A. Secondary structure of the Gag protein (NC domain) showing the two zinc knuckle motifs. B. Secondary structure of the HIV-1 (Ψ) packaging signal; DIS, dimer initiation site; SD, major splice donor site; gag, gag gene initiation sequence (from Amarasinghe et al, 2001).
Fig. 5. Alternative foldings of the HIV-1 leader. The retroviral untranslated leader consists of several functional domains. PolyA, polyadenylation domain; PAS, primer activation signal; PBS, primer binding site; DIS, dimer initiation site; SD, major splice donor site; \( \Psi \), core packaging signal; AUG, \( \text{gag} \) start codon. The polyA and DIS sequences are base paired to form the LDI structure. They form hairpins in the BMH structure. It is hypothesized that the BMH conformation exposes the DIS hairpin for subsequent dimerization (From Ooms et al., 2004).
Fig. 6. Secondary structure of the *Bovine leukemia virus* discontinuous encapsidation signal. The *gag* start codon is marked with a box (From Mansky and Wisniewski, 1998).
Fig. 7. Predicted secondary structure of the *Mouse hepatitis virus* 69-nucleotide packaging signal (From Fosmire et al., 1992).
Fig. 8. The predicted secondary structure of *Hepatitis B virus* (HBV) encapsidation (\( \gamma \)) signal, a bulged stemloop with the apical stem capped by a UGU (bold) tri-loop (From Flodell et al., 2002).
Fig. 9. Secondary structures of duck (DHBV) and heron (HHBV) hepatitis B virus encapsidation signals. Both are bulged stemloops. In DHBV, the upper stem is largely base paired, whereas HHBV upper stem is not (From Hu et al., 2004).
Fig. 10. Predicted structure of the packaging signal of *Flock house virus*. The essential packaging element is a 32-nucleotide bulged stemloop with a 5-nucleotide loop (From Zhong et al., 1992).
Fig. 11. The *Tobacco mosaic virus* origin of assembly. The OAS secondary structure is a bulged stem with a triplet G repeat (bold) terminal loop sequence (From Turner et al., 1988).
Fig. 12. Secondary structure of the *Turnip crinkle virus* essential element for packaging from 3752-3794 nucleotides. The packaging signal is a bulged stem loop (From Qu and Morris, 1997).
Fig. 13. The *Turnip yellow mosaic virus* 5' leader region with two hairpins containing protonatable internal loops (HP1 and HP2) that function as the packaging signal (From Bink et al., 2003).
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Chapter 3

The Packaging Scheme of Red Clover Necrotic Mosaic Virus Genomic RNAs

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The Packaging Scheme of *Red Clover Necrotic Mosaic Virus* Genomic RNAs

Abstract

*Red clover necrotic mosaic virus* (RCNMV) is a small icosahedral plant virus. While the RCNMV genome consists of two RNAs (RNA-1 and RNA-2), 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. RCNMV virions were subjected to UV crosslinking and heat treatment to determine the genomic RNA complement. A stable RNA-1: RNA-2 heterodimer was formed with either treatment. This finding establishes that the two genomic RNAs are co-packaged into a single virion. Furthermore, RNA-2 homodimers were also observed suggesting that some virions contain multiple copies of RNA-2 exclusively. These results indicate that the RCNMV population consists of two distinct types of virions: i) virions with both genomic RNAs in one; and ii) virions with multiple copies of RNA-2. Thus, these RCNMV packaging observations satisfy elements from both hypotheses.

Introduction

A crucial step in the life cycle of RNA viruses is the specific encapsidation of the viral genome by capsid protein (CP) subunits. The genome of an RNA virus can be either monopartite, such as the insect alphaviruses, or multipartite such as the
reoviruses. Typically, monopartite viruses are packaged into single particles whereas multipartite viruses can be encapsidated into either a single virion or multiple, distinct virions. For example, the genomes of reoviruses and certain bacteriophages are encapsidated into a single virion despite being multipartite (Patton and Spencer, 2000; Qiao et al., 1997). By contrast, the multipartite genome of bromoviruses utilizes a more complex packaging mechanism where the three genomic RNAs are encapsidated into three separate virions of identical size and morphology (Choi and Rao, 2003). Yet another RNA packaging mechanism exists for the bipartite *Flock house virus* (FHV) where each virion contains a copy of both RNA1 and RNA2 (Krishna and Schneemann, 1999; Selling and Rueckert, 1984).

Viral RNA genomes must be selectively recognized by the cognate CP during viral assembly. This recognition process occurs through a specific interaction between the viral CP and a genomic RNA packaging signal (Narayanan and Makino, 2001; McBride and Panganiban, 1996; Banks and Linial, 2000). Probably the most extensively characterized RNA packaging signal is that of *Tobacco mosaic virus* in which a 51-nucleotide stem loop element directs the specific assembly of the viral genome (Turner et al., 1988). Similarly, the specific packaging of the *Turnip crinkle virus* (TCV) genome was achieved by a 28-nucleotide RNA element (Qu and Morris, 1997). While these monopartite genomes are packaged into single virions, multipartite genomes face the additional problem of ensuring that all of the various genome segments are packaged.

*Red clover necrotic mosaic virus* (RCNMV) is a single-stranded positive-sense RNA plant virus of the *Dianthovirus* genus, family *Tombusviridae*. Each
RCNMV virion contains 180 subunits of the 37 kDa CP forming a non-enveloped, isometric particle of T=3 quasi symmetry, 30-35 nm in diameter (Lommel et al., 1988; Xiong and Lommel, 1989). The RCNMV genome consists of two RNAs, RNA-1 and RNA-2 (Fig. 1). The 3.9 kb RNA-1 codes for three proteins: i) p88, the RNA-dependent RNA polymerase, ii) p27, the replicase related protein and iii) the CP (Xiong et al., 1993b; Xiong and Lommel, 1989). The replicase proteins are translated directly from RNA-1 while the CP is expressed from a subgenomic RNA (sgRNA) (Zavriev et al., 1996). The 1.5 kb RNA-2 is monocistronic and codes for the movement protein (MP) required for cell-to-cell movement (Lommel et al., 1988; Osman et al., 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). This binding event is thought to produce the sgRNA via a premature termination mechanism (Sit et al., 1998; White, 2002).

The RNA complement of RCNMV virions has yet to be determined unequivocally. Purified virions form a single sharp band in CsCl density gradients suggesting that the population of virions displays a uniform density (Gould et al, 1981). However, analysis of purified virion RNA revealed a RNA-1: RNA-2 ratio of approximately 1:3 (S.A. Lommel, unpublished data). In addition, infectivity-dilution analysis for the related Carnation ringspot virus suggested the presence of two distinct types of particles in any given ratio (Hamilton and Tremaine, 1996). Based on the above observations, there are currently two main hypotheses for the
packaging of the RCNMV genome (Fig. 1B). The first (Fig. 1B, Model A) is based on the density gradient findings that suggest the co-packaging of both genomic RNAs into a single virion (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 results which suggest the presence of two distinct virions, each packaging either genomic RNA. The RNA-1 virion would contain a single copy of RNA-1 (3.9 kb) while the RNA-2 virion would contain three copies of RNA-2 (4.3 kb). The disparity in total RNA content between the two types of virions might not be distinguishable in CsCl density gradients. 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 (5.3 kb) while the other type would package four RNA-2 molecules, giving rise to virions with an RNA content of 5.8 kb.

Based on treatments employed in the structural studies of other icosahedral viruses, RCNMV virions were subjected to both UV irradiation and heat treatments to gain an insight into the true RNA complement within the virions. UV irradiation has been successfully used to determine the RNA complement within virions (Mayo et al., 1973; Newman and Brown, 1977). Heat treatment of Flock house virus (FHV) has been observed to result in the formation of an RNA complex between the two genomic RNAs suggesting a co-packaging arrangement of the RNAs within FHV virions (Krishna and Schneemann, 1999). RCNMV virions were subjected to both types of treatment. In each case, an RNA-1: RNA-2 heterodimer was formed. These treatments also produced an RNA-2 homodimer. These results are only possible if
the two RNAs are in close proximity. Thus, we can conclude that a proportion of the RCNMV virions co-package both RNA-1 and RNA-2 into a single virion while another fraction of the virion population packages RNA-2 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.

**Materials and methods**

**Plant inoculations**

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

**Virus purification**

Infected leaf tissue (0.5 g), exhibiting symptoms, was harvested four days post inoculation (d.p.i.). Virions were purified 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 minutes to pellet cell debris. The supernatant was subsequently filtered through Miracloth and virions were precipitated by the addition of ¼ 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 virions were transferred to a quartz cuvette and deoxygenated by the flow of N₂ gas while being stirred for 30 min. The sample was irradiated with UV radiation (255-300 nm) from a transilluminator (Fotodyne) for 20 minutes (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°C increments from 35-95°C to examine the role of temperature on the formation of RNA complexes.

**Virion RNA extraction**

Viral RNA was extracted from virions with phenol-chloroform after addition of SDS to 2% final concentration (Lommel et al, 1988). RNA in the aqueous phase was precipitated by the addition of 1/10 volume of 3 M sodium acetate, pH 4.8 and 2.5 volumes of 95% ethanol. The virion RNA was pelleted by centrifugation (13,500 g for 5 min) and resuspended in 10 µl of nuclease-free water.
**Gel electrophoresis of virion RNA**

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 at 90 V for 1 hour and visualized by staining with ethidium bromide.

For denaturing electrophoresis, 1 µl of viral RNA was electrophoresed through a 1% agarose gel containing 1.8% formaldehyde in 1X MOPS (morpholinopropane sulfonic acid, pH 7.0) buffer. The RNA was visualized by staining with ethidium bromide.

**Northern blotting**

Electrophoresed viral RNA was blotted to Magnaprobe™ membranes (Osmonics) by capillary transfer in 5X SSC (sodium chloride-sodium citrate) buffer. The membrane was then subjected to optimal UV crosslinking with the Stratalinker® (Stratagene). 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). 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).
**RNase T₁ digestion of heat treated virions**

Aliquots of heated and unheated virions (30 µg) were treated with 1 U RNase T₁ (Ambion) at room temperature for 15 min. After nuclease digestion the samples were digested with Proteinase K and virion RNA was extracted as mentioned above.

**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 MgCl₂, 10 mM Tris-HCl, pH 7.5, 40 mM NaCl) followed by slow cooling to room temperature (Dirac et al., 2002). Purified virion RNA was also heat treated but only as a combination of both RNAs. 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 50,000X magnification.

**Results**

**An RNA-1: RNA-2 heterodimer is produced upon UV irradiation of RCNMV virions**

Irradiation of two RNAs by far UV light results in crosslinking only when both RNAs are in close proximity through either transient contacts or base pairing interactions. These RNA crosslinks are referred to as zero-length crosslinks due to
the relative spatial proximity to each other. The RNA crosslinks are formed by covalent interactions and are stable under denaturing conditions.

UV irradiation of *Raspberry ringspot virus* (RRSV) was used to induce the formation of RRSV RNA-2 homodimers due to the RNAs being packaged within the same particle (Mayo et al., 1973). This led to the conclusion that two RNA-2 molecules were packaged within one virion while RNA-1 was packaged into a separate virion.

UV irradiation of RCNMV virions resulted in the formation of an RNA species ~5.3 kb in length under non-denaturing conditions (Fig. 2). Bands corresponding to RNA-1 and RNA-2 (3.9 and 1.5 kb, respectively) were also observed. Northern analysis of this gel revealed the 5.3 kb species to be a heterodimer of RCNMV RNA-1 and RNA-2. Additional bands corresponding to multimers of RCNMV RNA-2 (~ 3.0 and 4 kb) were also observed (Fig. 2). Since UV irradiation of virions only induces crosslinking between RNAs that are in close contact, the formation of hetero- and homodimers is proof that RCNMV virions contain both RNA-1 and RNA-2 or just RNA-2.

To investigate whether the RNA-1: RNA-2 heterodimer produced by UV irradiation is a covalently-linked product, the viral RNA extracted from UV-irradiated virions was subjected to denaturing gel electrophoresis prior to northern analysis. Under denaturing conditions, the hetero- and homodimers persisted (Fig 4). Thus, the respective crosslinks induced by UV irradiation appear to be stable due to covalent linkage.
An RNA-1: RNA-2 heterodimer is also formed upon heat treatment of virions

Based on the novel heating approach employed by Krishna and Schneemann (1999) for heterodimer formation within FHV virions, RCNMV virions were treated similarly. Like RCNMV, FHV contains a bipartite genome encapsidated within an icosahedral virion of similar size. Purified RCNMV virions heated at 65°C generated an RNA species approximately 5.3 kb in size which is larger than either genomic RNA (Fig. 3). This larger RNA species is the size of RNA-1 and RNA-2 combined. To verify the composition of this larger RNA species, northern analysis was performed on the heat-treated viral RNA. Duplicate blots were processed separately with RNA-1 and RNA-2 specific probes. The 5.3 kb RNA species hybridized to both RNA-1 and RNA-2 specific probes indicating that this species is a heterodimer complex consisting of both genomic RCNMV RNAs (Fig. 3). Therefore, the heating of RCNMV virions at 65°C produces a similar result to that observed in FHV, being the formation of a heterodimer complex between RNA-1 and RNA-2. This heterodimerization can only occur when the RNAs are in close proximity, indicating that a portion of the RCNMV virion population co-packages RNA-1 and RNA-2 into the same virion.

Based on inspection of ethidium bromide stained gels, approximately 25% of the virion RNA was present as the 5.3 kb heterodimer complex. Northern analysis revealed the presence of additional intermediate RNA species consisting solely of RNA-2. These RNA species are approximately 3 and 4.5 kb in size which would correspond to 2 and 3 copies of RNA-2, respectively. Thus, heating of RCNMV virions appears to generate not only heterodimers but also RNA-2 homodimers and
multimers. The formation of these RNA-2 species suggests that a portion of the RCNMV virion population packages RNA-2 exclusively.

The above findings confirm and expand on the results obtained by UV irradiation of virions. Namely, RCNMV virions either co-package both genomic RNAs into a single virion or package RNA-2 solely.

**Formation of the RNA-1: RNA-2 heterodimer is temperature dependent**

To further define the conditions for RNA complex formation, experiments were performed to determine the temperature range at which heterodimer formation occurs (Fig. 5). Stable heterodimer complexes were observed in virions treated at temperatures between 45-85°C. Optimal heterodimer accumulation was observed at 65°C with decreasing efficiencies of formation above and below this temperature. At 45°C and 85°C, heterodimer formation was 10% of the optimal heterodimerization observed at 65°C. It is not known whether this temperature effect is related to heterodimer formation or heterodimer stability.

**The RNA-1: RNA-2 heterodimer is formed within virions by means of a non-covalent interaction**

Despite being heated at 65°C, RCNMV virions appear to be intact (Fig. 6). However, this does not preclude the possibility that the genomic RNA is extruded prior to complex formation. To verify that heterodimer complex formation occurs within the virion, heated and unheated virions were treated with RNase T₁ prior to viral RNA extraction. The RNA heterodimer complex formed during heating persisted despite the nuclease treatment (Fig. 6). As expected, the non-heated virions did not generate the heterodimer complex and were not susceptible to RNase T₁.
degradation. This illustrates that the heat treatment does not expose the virion RNA to the exterior and that dimerization occurs exclusively within the protected environment of the virion.

The nature of the interaction between RNA-1 and RNA-2 in the heterodimer complex was determined by subjecting the heat-treated viral RNA to denaturing gel electrophoresis. The heterodimer complex did not persist under denaturing conditions as observed by northern analysis (Fig. 4). This reveals that the heterodimer formed during heat treatment was held together by non-covalent interactions.

**RCNMV RNA does not form dimer complexes upon heating *in vitro***

Viral RNA dimer formation plays an integral role in the packaging of the *Human immunodeficiency virus*-1 (HIV-1) genome (Skripkin et al., 1994). Retrovirus RNA transcripts formed dimers when heated to 65°C (Dirac et al., 2002). The addition of nucleocapsids was shown to increase the dimer formation efficiency for HIV-1 transcripts *in vitro* in a manner suggestive of the native mechanism for assembly (Skripkin et al., 1994). As a first step in determining whether dimer formation is an integral event in the RCNMV virion assembly process, RCNMV RNA transcripts as well as virion RNA were annealed *in vitro* in the absence of CP. No homodimers of either RNA-1 or RNA-2 were ever observed when RCNMV transcripts were annealed *in vitro* (Fig. 7). Similarly, no RNA-1: RNA-2 heterodimers were ever detected upon annealing of either genomic RNA transcripts or virion RNA (Fig. 7). 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.7).

Discussion

The formation of genomic RCNMV RNA dimers occurring within the confines of the virion shell clearly shows that at least two RNAs are co-packaged per RCNMV virion. Regardless of the treatment involved (either UV-irradiation or heating) the same spectrum of RNA multimer species were generated. These include RNA-1: RNA-2 heterodimers, RNA-2 homodimers and RNA-2 multimers. 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 infectivity-dilution assays for dianthoviruses which have suggested 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 sharp 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 (5.3 kb), it is likely that RNA-2 only virions contain four copies of RNA-2 (5.9 kb) (Fig. 1B). This
difference in total RNA content per virion may not manifest itself in an observable difference in density on a CsCl gradient.

UV irradiation of RCNMV virions was the first method employed to characterize the RNA complement within virions. UV-crosslinking of RNAs is a powerful tool used for the determination of the spatial organization of RNAs within macromolecular structures (Branch et al., 1985; Circle et al., 2003; Prince et al., 1982; Yaniv et al., 1969; Zhirnov and Wollenzien, 2003; Zwieb et al., 1978). There have been prior instances where UV irradiation-induced crosslinking was used to determine the RNA content within virions. Newman and Brown (1977) UV irradiated Nodamura virus, which is similar to RCNMV in genome size, organization and particle architecture. Irradiated virions displayed reduced infectivity according to single hit kinetics suggesting that the genomic RNAs were contained in a single virion. A reduction of the individual RNA components was observed suggesting that dimer formation was present but undetectable with the techniques used in this study. However, the two genomic RNAs, present in equimolar amounts, were released as a native complex upon treatment with guanidium and low concentrations of SDS (Newman and Brown, 1977). Unlike Nodamura virus, the genomic RNAs of the related FHV are not observed as a pre-formed native complex (Krishna and Schneemann, 1999). The RCNMV RNA-1: RNA-2 heterodimer is observed only after UV irradiation.

While UV irradiation was used to characterize Nodamura virus, heat treatment of virions was utilized to characterize the RNA complement within FHV virions (Krishna and Schneemann, 1999). Similar to UV irradiation, heat treatment
also produced a slight reduction in infectivity although no morphological changes to the virions were observed (Krishna and Schneemann, 1999). Heat treatment of RCNMV virions also leads to RNA-1: RNA-2 heterodimer formation without any apparent changes in virion morphology (Fig. 6). Rearrangement of virion RNA due to heating has been observed for FHV, which, when heated to 65°C, extrudes the RNA from a single point in the virion (Cheng et al., 1994). Although rearrangement of RCNMV RNA within virions may have occurred, extrusion of RNA from virions was not observed upon exposure to 65°C. 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 *N. benthamiana* (data not shown). The different responses of the viral RNAs to heat treatment (FHV extrudes RNA while RCNMV does not) may reflect differences in their respective disassembly mechanisms.

The formation of RNA dimers within virions is not a phenomenon unique to Nodaviruses and RCNMV. Genomic homodimers occur naturally and are a requirement for HIV-1 encapsidation (Laughrea et al., 1997; Sakuragi et al., 2003). HIV-1 RNA dimers are preferentially packaged while monomeric RNAs are excluded (Fu et al., 1994; 1990; Fu and Rein, 1993). 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 an 8-nucleotide
element (termed the TA binding site or TABS) within the RNA-1 CP subgenomic promoter (Sit et al., 1998; Fig. 1). 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 functional similarity between the TA-TABS structure of RCNMV and the HIV-1 kissing loop. The DIS of HIV-1 is GC-rich (GCGCGC) as is the TA element of RCNMV (CGCCCC). Thus, the TA-TABS interaction may function similarly to the HIV-1 DIS to ensure dimer formation between RCNMV RNA-1 and RNA-2 for co-packaging.

Initiation of CP synthesis is the primary purpose of the RCNMV TA-TABS interaction. Due to the genetic compactness of the RCNMV genome, this interaction also provides an ideal intermediate in the assembly process since both genomic RNAs are in close proximity. Formation of the TA-TABS structure is favored late in the viral life cycle when levels of both genomic RNAs are high enough for virion production. In HIV-1 and *Moloney murine leukemia virus* (MoMuLV), dimerization causes a rearrangement of the RNA exposing ψ to initiate encapsidation (D’Souza and Summers, 2004; Huthoff and Berkhout, 2001). In RCNMV, the TA-TABS interaction may play a similar role in the viral life cycle by forming or exposing the CP binding site. Since the TA-TABS structure forms prior to assembly, it is tempting to speculate that this structure catalyzes the dimerization of genomic RCNMV RNA.

Based on non-denaturing gel electrophoresis, the RCNMV heterodimer complex is formed by a non-covalent interaction. Interestingly, in HIV-1, heat treatment was shown to melt the intra-strand base pairs of the DIS stem and induce
the formation and extension of the dimer (Laughrea and Jette, 1996; Muriaux et al., 1996b). Genomic dimers of HIV-1 were found to be too labile to survive electrophoresis on TBE gels but heating of the virions to temperatures of 50 to 60°C yielded stable dimers (Clever et al., 1996; Feng et al., 1996). Immature HIV-1 virions contain the genomic homodimer in the loose form and it is converted to a tight dimer upon maturation which is essential for its infectivity (Feng et al., 1996).

Attempts to generate RCNMV RNA-1: RNA-2 heterodimers \textit{in vitro} utilizing either RNA transcripts or purified RNA proved unsuccessful. Transcripts of HIV-1 have been shown to spontaneously dimerize \textit{in vitro} (Darlix et al., 1990; Feng et al., 1996). HIV-1 nucleocapsid protein has been demonstrated to act as a chaperone which enhanced homodimer formation (Feng et al., 1996; Muriaux et al., 1996a).

The current annealing conditions employed for RCNMV RNA may require optimization through the addition of other compounds such as CP whose absence could be a limiting factor.

Although the TA-TABS interaction is proposed to be the initial contact point for dimerization, it is not beyond the realm of possibility that additional points of interaction between RCNMV RNA-1 and RNA-2 exist. Long stretches of significant complementarity between RNA-1 and RNA-2 do not exist. Interestingly, a sequence complementary to the TA element is located in the 3’ region of RNA-2 between nucleotides 1303-1308 (Fig. 8). This six-nucleotide element (GGGGUU), termed the TABS mimic (TABS\textsubscript{M}), is also conserved in the Canadian and Czechoslovakian strains of RCNMV as well as two strains of \textit{Sweet clover necrotic mosaic virus}. A \textit{trans} interaction between the TA and TABS\textsubscript{M} of opposing RNA-2 molecules may
lead to homodimer formation. This specific interaction may be important for homodimer formation. However, 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 homodimers of RNA-2 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 heterodimer containing ones. There is precedence for this supposition based on SDS treatment of *Carnation ringspot virus* (Tremaine and Ronald, 1976). These studies illustrated the preferential liberation of RNA-2 from particles at low SDS concentrations prior to RNA-1 release at higher SDS concentrations. Thus, formation of tetrameric RNA-2 upon UV irradiation or heating may not have been favored whereas dimeric and trimeric forms of RNA-2 were more likely.

Based on heterodimer and homodimer formation, we hypothesize that RCNMV infections produce a population containing at least two forms of virions: i) one that co-packages one copy each of RNA-1 and RNA-2 and ii) one that contains four copies of RNA-2. This packaging arrangement for a multipartite RNA genome with icosahedral virions is unique among both plant and animal viruses.
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Fig. 1. Red clover necrotic mosaic virus genome organization and various possibilities for packaging the genomic RNAs. A. Schematic representation of the RCNMV genome. The ORFs are depicted as boxes and the encoded products are indicated within the boxes. The protein products, p27 and p88 are replicase proteins, CP is the capsid protein and MP the movement protein. The position of the ribosomal frameshift event is indicated which is responsible for the translation of p88. B. Various possibilities for packaging of RCNMV genomic RNA-1 and RNA-2 into virions. Model A, only one virion type produced with one copy of RNA-1 and one copy of RNA-2 together in a single virion and only one virion population. Model B, two virion populations, one with one copy of RNA-1 and the other with 3 copies of RNA-2. Model C, a different type of two virion populations, one with one copy each of RNA-1 and RNA-2 and the other type with 4 copies of RNA-2.
Fig. 2. UV crosslinking of RCNMV virions. RCNMV virions (two different aliquots, 50 and 100 µg) were UV irradiated at 255-300 nm for 20 min. UV-crosslinked virions were Proteinase K digested and virion RNA was purified. Gel electrophoresed virion RNA was analyzed by northern blotting. Asterisks denote the dimers formed. A single asterisk indicate the RNA-1: RNA-2 heterodimer, the double asterisk indicate the RNA-2 homodimer whereas the triple asterisk indicate the RNA-2 multimer of 3 RNA-2 molecules. T7 RNA transcripts of the respective genomic served as controls. The molecular weights of the RNA and the complexes are given to the right of the blots.
Fig. 3. Gel electrophoresis of virion RNA purified from heated and unheated RCNMV virions. Virions were heated to 65°C for 10 min followed immediately by quenching on ice. Unheated virions were kept at 4°C. Phenol chloroform purified RNA was electrophoresed through a non-denaturing 1% agarose-TAE gel and analyzed by northern hybridization. T7 RNA controls of each genomic RNA served as controls. RNA size marker is in nucleotides. Single asterisk indicates signal indicating the formation of an RNA-1:RNA-2 heterodimer. The double asterisk indicates the presence of an RNA-2 multimer of three molecules. Molecular weights are indicated to the right of the blots.
Fig. 4. Denaturing gel electrophoresis and northern analysis of heat treated and UV-crosslinked RCNMV virion RNA. Virion RNA extracted from both heat treated and UV-crosslinked RCNMV virions were electrophoresed on a 1% agarose-formaldehyde gel and analyzed by northern hybridization. T7 RNA transcripts were included as controls. The heterodimers and the homodimers are denoted by the asterisks. The molecular weights are indicated to the right of blots.
Fig. 5. Temperature range for RCNMV RNA complex formation and effects of heating on virion morphology. A. The temperature range of the heterodimer formation. Virions were heated to a range of different temperatures and virion RNA was extracted and gel electrophoresed on a 1% agarose-TBE gel. The RNA-1: RNA-2 heterodimer is indicated in an asterisk and the molecular weight is indicated to the right of the gel. Respective RNA molecules are indicated to the right. B. EM images of heated and unheated RCNMV virions. RCNMV virions were heated at 65°C for 10 min and laid on copper grids, stained with 2% uranyl acetate and observed under EM. Unheated RCNMV virions are depicted to the left whereas the heated virions are towards right. Scale bar is 100 nm.
Fig. 6. Rnase T1 treatment of heated and unheated virions. Virion samples (heated to 65°C or unheated) were treated with Rnase T1 and incubated at room temperature for 15 min (+). Purified virion RNA was treated with Rnase T1 as a positive control. All of the above samples were incubated at room temperature without Rnase T1 as negative controls (-). After incubation all samples were digested with proteinase K for 30 min at 37°C followed by phenol chloroform purification. RNA was gel electrophoresed on a 1% agarose-TBE gel and visualized with ethidium bromide. The RNA-1: RNA-2 heterodimer is indicated with an asterisk.
Fig 7. Heating of RCNMV transcripts and virion purified RNA. Transcripts of both RNA-1 and RNA-2 as well as virion purified RNA was heated to 65°C for 10 min followed by immediate cooling. Gel electrophoresis of the heated RNA as well controls were done in a 1% non-denaturing agarose gel. RNA-1 and RNA-2 are indicated. Note that no RNA complex is formed.
Fig. 8. A comparison of the TA, TABS and $\text{TABS}_m$ sequences. The RNA-2 TA sequence which is complementary to the RNA-1 TABS forms a base-pairing interaction resulting in CP sgRNA synthesis. The additional TA complementary sequence, $\text{TABS}_m$ resides within the 3' region of RNA-2. The 6 nt $\text{TABS}_m$ element shares a 5 nt similarity with the TABS (shaded). There may also be an additional non-Watson-Crick base pairing involved in the TA-$\text{TABS}_m$ interaction.
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Chapter 4

The Red Clover Necrotic Mosaic Virus Origin of Assembly: A Third Function for the RNA-2 Trans-Activator

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The Red Clover Necrotic Mosaic Virus Origin of Assembly: A Third Function for the RNA-2 Trans-Activator

ABSTRACT

The bipartite single-stranded (+) sense RNA genome of Red clover necrotic mosaic virus (RCNMV) is packaged by a single species of capsid protein (CP) into 30-35 nm icosahedral particles with T=3 symmetry. RNA-1 codes for the viral polymerase and the CP while RNA-2 codes for the movement protein (MP). We have previously shown that RCNMV exists as two populations: i) virions that co-package RNA-1 and RNA-2 and ii) virions which contain multiple copies of RNA-2 (Chapter 3). To identify the RCNMV origin of assembly, mutations were engineered on RCNMV genomic RNA and tested for packaging efficiency using an in vivo infectivity assay. The RNA content within RCNMV virions displays a narrow size range. We have determined that RNA-1 does not contain a discrete, independent packaging signal. Furthermore, we show that the CP subgenomic RNA (sgRNA) is not encapsidated into RCNMV virions. On RNA-2, a 209 nucleotide region within the MP open reading frame was identified as being essential for RCNMV packaging. Further deletion analysis indicated that the 34 nucleotide trans-activator element, found within this region, is the origin of assembly for RCNMV. Consequently, to package RNA-1 it must at least be base-paired with RNA-2 at the trans-activator. This RNA-2 multifunctional stem loop structure was previously shown to: i) direct the synthesis of CP sgRNA from RNA-1 and ii) serve as a cis-acting replication element.
for RNA-2 accumulation. The addition of viral assembly duties illustrates the critical importance of the trans-activator element as a key regulatory switch in the RCNMV life cycle.

INTRODUCTION

Virion assembly is directed by RNA-protein and protein-protein interactions (Muriaux et al., 2001; Zlotnick, 2003). Assembly initiation proceeds with the binding of capsid protein (CP) subunits to a specific RNA structure to form the initiation complex. This nucleic acid-protein interaction is followed by the nucleation of additional CP subunits to form the completed virion (Fox et al., 1994). Formation of the initiation complex is determined by a specific RNA sequence(s) and/or structural element(s) known as the origin of assembly sequence (OAS) or packaging signal that is involved in binding of the cognate viral CP. This specificity discriminates against the packaging of heterologous cellular RNAs.

Packaging signals for numerous animal viruses with differing virion morphologies have been identified (Alanen et al, 1987; Banks and Linial, 2000; Beasley and Hu, 2002; Doria-Rose and Vogt, 1998; Makino et al, 1990; McBride and Panganiban, 1996; Weiss et al, 1994; Yang and Temin, 1994). All characterized OASs form simple secondary structural elements, such as stem loops, which can function in a heterologous context. The structure is the primary packaging determinant but sequence is the overriding factor for packaging specificity in some cases (Turner et al., 1988). Most progress has been made with retroviruses,
especially Human immunodeficiency virus-1 (HIV-1), due to the targeting of viral assembly for antiviral therapy (reviewed in Muriaux et al., 2004). The essential encapsidation signal for HIV-1 is a stem loop with a 4 nt terminal loop (Luban and Goff, 1994; McBride and Panganiban, 1997) but optimal assembly requires the encompassing regions to be folded into a highly ordered structure (De Guzman et al., 1998; Amarasinghe et al., 2001).

Several plant virus OASs have been identified with the most extensively characterized one being that of the rod-shaped Tobacco mosaic virus (TMV; Zimmern, 1983; Turner and Butler, 1986; Turner et al., 1988). The TMV OAS is a 51 nt bulged stem loop with a G residue at every third nucleotide in the terminal loop. Each CP subunit binds to three nucleotides and to the G residues of the loop preferentially to initiate assembly (Turner et al., 1988). As definitive proof of OAS determination, TMV CP was shown to direct efficient packaging of unrelated and chimeric RNA containing the TMV OAS (Gallie et al., 1987; Sleat et al., 1986; Turner et al., 1989). Less is known regarding icosahedral plant RNA virus assembly and their packaging signals. Turnip crinkle virus (TCV) a member of the Carmovirus genus, family Tombusviridae, is the only small icosahedral plant RNA virus for which the OAS is reasonably well characterized. TCV produces a 30 nm virion which packages a monopartite genome of approximately 4.0 kb. A 28 nt bulged hairpin loop was identified as the essential packaging element (Qu and Morris, 1997). Considerable characterization of the Brome mosaic virus (BMV) OAS has also been conducted. Although a specific signal has not been characterized, it has been demonstrated that the conserved 3’ tRNA-like structure (TLS) of the genomic RNA
facilitates assembly (Choi et al., 2002). An additional upstream signal was identified as a requirement for packaging along with the TLS (Choi and Rao, 2003; Damayanti et al., 2003).

Red clover necrotic mosaic virus (RCNMV) is a small icosahedral virus of the genus Dianthovirus, family Tombusviridae. Dianthoviruses are taxonomically distinct from other genera in the family due to the bipartite nature of its single-stranded, positive-sense RNA genome. RCNMV produces approximately 30-35 nm isometric virions composed of 180 copies of the 37 kDa CP subunit arranged in a T=3 icosahedron. The two genomic RNAs, RNA-1 and RNA-2 (3.9 kb and 1.45 kb, respectively; Fig. 1)) are non-homologous. RNA-1 codes for three proteins: i) the 88 kDa RNA dependent RNA polymerase (p88), ii) the 27 kDa protein (p27) with unspecified, but probable replicase function and iii) the 37 kDa CP (Kim and Lommel, 1994; Xiong et al., 1993b; Xiong and Lommel, 1989). The CP open reading frame (ORF) is 3' proximal and is translated from a subgenomic RNA (sgRNA; Osman and Buck, 1990; Zavriev et al., 1996). RNA-1 can replicate independently but cannot move from cell-to-cell in the absence of RNA-2 (Osman and Buck, 1987; Paje-Manolo and Lommel, 1989; Xiong et al., 1993a) which codes for the 35 kDa movement protein (MP; Lommel et al., 1988). Although CP is not required for cell-to-cell movement, CP in the form of virions is hypothesized to be required for systemic transport through the vasculature (Vaewhongs and Lommel, 1995; Xiong et al., 1993a). Subsequently, both RNA-1 and RNA-2 are required for a successful infection. It was discovered that RNA-2 plays a key role in the regulation of CP expression from RNA-1 (Sit et al., 1998). The trans-activator (TA) element, a 34 nt
stem loop structure within the MP ORF, \textit{trans}-activates CP sgRNA transcription from RNA-1 by basepairing with the TA binding site (TABS) found within the RNA-1 CP subgenomic promoter. Structural probing of the TA-TABS interaction demonstrated the formation of a stacked helical structure (Guenther et al., 2004). Thus, this RNA-1: RNA-2 interaction is a critical step in the assembly pathway for RCNMV.

Multipartite viruses display diverse mechanisms for packaging of their genomic RNAs. BMV is an icosahedral plant RNA virus with a multipartite genome that produces three distinct types of particles packaging different combinations of its genomic and sgRNA. BMV packages RNA1 and RNA2 into separate virions while co-packaging RNA3 and its sgRNA (RNA4) into a single virion (Loesch-Fries and Hall, 1980; Choi et al., 2002). This ordered packaging arrangement of BMV is achieved by the presence of a genome specific element on each RNA (the TLS) combined with a segment specific element to form the complete packaging signal (Choi and Rao, 2003; Damayanti et al., 2003).

\textit{Flock house virus} (FHV) is a small icosahedral insect virus with a bipartite genome akin to RCNMV. Both FHV RNA1 and RNA2 are co-packaged into a single virion with the packaging signal identified as a 32 nt stem loop structure residing on RNA2 (Newman and Brown, 1977; Selling and Rueckert, 1984; Krishna and Schneemann, 1999; Zhong et al., 1992). Although both FHV RNAs are co-packaged, it has not been determined how both RNAs interact to become packaged into a single virion when RNA-2 contains the cognate packaging signal. FHV RNAs form a heterodimer upon heating leading to the hypothesis that heterodimer
formation enables FHV CP to co-package both RNAs (Krishna and Schneemann, 1999).

Analysis of previous observations on the RNA complement of RCNMV suggested the presence of a complex, non-uniform and possibly novel packaging strategy for RCNMV. CsCl density gradient studies indicated that all RCNMV virions are of equal density (Gould et al., 1981; Hollings and Stone, 1977) suggesting that the two genomic RNAs are co-packaged. However, RNA liberated from purified virions exhibits a ratio of approximately 1:3 of RNA-1: RNA-2 (S.A. Lommel, unpublished data). To determine the packaging scheme of RCNMV, virions were subjected to both heating and UV-irradiation (Chapter 3). Our results suggest that RCNMV produces two species of virions: i) one species that co-packages RNA-1 and RNA-2 and ii) a second species with multiple copies of RNA-2. Although RNA-1 may contain a unique packaging signal, it is likely that RNA-2 contains the discrete, independent and fully functional OAS for RCNMV since all virions must contain RNA-2 and some virions contain only RNA-2.

A system for the efficient *in vitro* assembly of RCNMV virions has never been developed forcing us to utilize *in planta* infectivity assays to study RCNMV packaging. Here we report the identification of the OAS for RCNMV using this *in vivo* approach. We utilized two assay systems to determine the RCNMV OAS: i) the heterologous TBSV vector (pHST2) delivering fragments of RNA-2 and ii) deletion mutations directly on RCNMV RNA-2. In both cases, packaging capabilities were determined by co-inoculation with wild-type RNA-1. We demonstrate that a 209 nt sequence within the MP coding region of RNA-2 was able to direct packaging of the
heterologous TBSV RNA in the presence of RNA-1. M-FOLD secondary structure analysis of this 209 nt sequence revealed a highly folded secondary structure of five stem loops, one being the TA element. Further deletion analysis of these putative stem loops directly on RNA-2 revealed that the TA element was the critical structure for virion assembly. As definitive evidence that the RCNMV packaging signal is the RNA-2 TA element, RCNMV CP was able to produce wild type virions packaging a heterologous TBSV RNA containing the TA element. The TA element was previously shown to be a critical factor in the trans-activation of transcription (Sit et al., 1998) and recently it was determined to be a cis –acting replication element (Tatsuta et al., 2005). The additional role for the TA as the distinct packaging signal of a bipartite plant RNA virus confirms the TA as a critical multifunctional element in the RCNMV life cycle.

**MATERIALS AND METHODS**

**Plasmid constructs.** Synthesis of infectious full-length cDNA clones of RCNMV RNA-1 (pRC1) and RNA-2 (pRC2) as well as the sGFP expressing RNA-1 construct (R1sGFP) were described previously (Sit et al., 1998; Xiong and Lommel, 1991).

**(i) pHST2-based constructs.** The TBSV construct pHST2 (kindly provided by H.B. Scholthof, Texas A&M) served as the heterologous vector for the expression of RCNMV RNA-2 sequences. Constructs pHST2-ΔBX and –SL2 (containing 209
and 34 nt, respectively, from RCNMV RNA-2) were described previously (Sit et al., 1998).

pHST2-△BA was derived from pHST2-△BX by cleavage with Xhol/AgeI followed by blunting with Klenow fragment and re-ligation to yield a construct with a 400 nt deletion.

pHST2-△BP was derived from construct pHST2-△BX by the deletion of 260 nt from the TBSV vector as follows. A PCR fragment was first amplified with PfuTurbo® DNA polymerase (Stratagene) from pHST2 utilizing primers pHST2 2941 X/B and pHST2 -3475 followed by cleavage with Xhol/NcoI. Subsequently, pHST2-△BX was digested with Xhol/NcoI followed by ligation of the pHST2 PCR fragment to generate pHST2-△BP.

pHST2-SL△NA was derived from pHST2-SL2 by cleavage with NotI/AgeI followed by blunting with Klenow fragment and re-ligation to yield a construct with a 390 nt deletion.

pHST2-RCP is a construct that expresses a complete copy of the RCNMV CP ORF. The RCNMV CP ORF was amplified by PCR with Taq DNA polymerase (New England Biolabs) using oligos; 5’ RCP and 3’ RCP. The PCR fragment was subcloned into the pGEM-T vector (Promega) and subsequently isolated after cleavage with Xhol/Stul. The pHST2 vector was cleaved with Xhol/SnaBI and dephosphorylated with calf intestinal alkaline phosphatase prior to ligation of the CP insert.

(ii) RNA-2 mutants. Site-directed mutants of RCNMV RNA-2 were synthesized from pRC2 utilizing the QuikChange® site-directed mutagenesis kit.
The oligonucleotides used in the construction of the mutants were synthesized by MWG Biotech and listed in Table 1.

R2Δg1-g3 contains a deletion of all structural elements characterized within the 209 nt region of RCNMV RNA-2. An EcoRI site was introduced into pRC2 at nt 730 by site-directed mutagenesis with the oligonucleotide MP214 ECO STOP to generate MP214S1. This resultant construct was cleaved with EcoRI/BglII followed by blunting with Klenow fragment and re-ligation to yield a construct with a 186 nt deletion.

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

**Virus purification.** Infected leaf tissue (0.5 g), exhibiting symptoms, was harvested four days post inoculation (d.p.i.). Virions were purified 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 minutes to pellet cell debris. The supernatant was subsequently filtered through Miracloth and virions were precipitated by the addition of ¼ 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 \( \mu l \) 10 mM Tris-HCl, pH 6.5.

**Virion RNA extraction and RT-PCR.** Viral RNA was extracted from virions with phenol-chloroform after addition of SDS to 2% final concentration (Lommel et al, 1988). RNA in the aqueous phase was precipitated by the addition of 1/10 volume of 3 M sodium acetate, pH 4.8 and 2.5 volumes of 95% ethanol. The virion RNA was pelleted by centrifugation (13,500 \( g \) for 5 min) and resuspended in 10 \( \mu l \) of nuclease-free water.

RCNMV virion RNA was analyzed for TBSV sgRNA packaging by RT-PCR. First strand synthesis was initiated with SuperScript™ II reverse transcriptase (Invitrogen) and pHST2 -3475 primer according to the supplier’s instructions. The cDNA was amplified with GoTaq DNA polymerase (Promega) utilizing primers RC2 TA CLA 5’ (+) and pHST2 -3475.

**Total Leaf RNA extraction.** Total RNA was purified from 100 mg of *N. benthamiana* leaves 4 d.p.i. utilizing the RNeasy® Plant Mini Kit (Qiagen) according to the manufacturer’s protocol. RNA was resuspended in 50 \( \mu l \) of nuclease-free water.

**Gel electrophoresis.** For non-denaturing electrophoresis, a 1 \( \mu l \) aliquot of purified viral RNA or total RNA was electrophoresed through a 1% agarose gel in Tris-acetate EDTA buffer at 90 V for 1 hour and visualized by staining with ethidium bromide.
For semi-denaturing electrophoresis, a 1 µl aliquot of purified viral RNA or total RNA was electrophoresed through a 1% agarose gel in Tris-borate EDTA buffer at 90 V for 1 hour and visualized by staining with ethidium bromide.

For denaturing electrophoresis, 1 µl of viral RNA or total RNA was heated at 65°C for 3 min prior to electrophoresis through a 1% agarose gel containing 1.8% formaldehyde in 1X MOPS (morpholinopropane sulfonic acid, pH 7.0) buffer. The RNA was visualized by staining with ethidium bromide.

**Northern blotting.** Electrophoresed viral RNA was blotted to Magnaprobe™ membranes (Osmonics) by capillary transfer in 5X SSC (sodium chloride-sodium citrate) buffer. The membrane was then subjected to optimal UV crosslinking with the Stratalinker® (Stratagene). DNA probes corresponding to the RNA-1 CP open reading frame (ORF) and the RNA-2 MP ORF were labeled with 32P-dCTP via random primer labeling (Rediprime II Random Prime Labeling System, Amersham Biosciences). 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).

**Western blotting.** Total leaf proteins were extracted from *N. benthamiana* tissue (0.5 g each) according to the protocol described by Petty et al. (1989). Samples were electrophoresed by SDS-PAGE through 12% Ready Gels® (BioRad).
at 200 V for 50 min in Laemmli running buffer (Laemmli, 1970). Proteins were transferred to nitrocellulose membranes (GE Osmonics) in transfer buffer (25 mM Tris, 40 mM glycine) utilizing a BioRad Trans-Blot® SD transfer cell. After transfer, the membrane was blocked in 50 ml TBST buffer (50 mM Tris, 0.15 M NaCl, 0.05% Tween-20, pH 7.4) containing 3% low fat dry milk. RCNMV CP was detected with rabbit polyclonal antisera raised against RCNMV virions, followed by incubation with goat anti-rabbit horseradish peroxidase conjugated antisera (Sigma). The protein bands were visualized by the ECL™ western blot detection kit (GE Healthcare) as directed by the manufacturer and exposed to Kodak Chemiluminescence MS film.

**Electron microscopy.** The purified virions were applied to Formvar-coated copper grids, stained with 2% uranyl acetate and blotted dry. Virus preparations were visualized with a JEM-100 S transmission electron microscope (EM) at 50,000X magnification.

**RESULTS**

**RCNMV sgRNA is not packaged into RCNMV virions.** Based solely on the size of the RCNMV CP sgRNA, the possibility exists that it may be co-packaged with multiple copies of RNA-2. Although, there is no previous data to support the packaging of the RCNMV sgRNA, examples exist of other multipartite plant viruses which do package their sgRNAs. BMV is one such example where RNA-3 is co-packaged with the CP sgRNA (RNA4; Loesch-Fries and Hall, 1980; Rao, 2001). To better characterize the RCNMV assembly process, we needed to rule out the
possibility that the sgRNA was packaged into virions. Viral RNA was extracted from RCNMV virions and analyzed for RNA content. Viral RNA was hybridized to probes from RNA-1 specific for the 5’ p27 ORF or the 3’ CP ORF (Fig 2). Both probes hybridized to genomic RCNMV RNA-1 as expected. However, there was no distinct signal at the position corresponding to the sgRNA with the CP ORF probe. Thus, we conclude that the CP sgRNA is not packaged into RCNMV virions.

**RNA-1 does not package into virions in the absence of RNA-2.** We produced a TBSV construct to express the RCNMV CP to uncouple the requirement of RNA-2 trans-activation for CP synthesis. This construct, pHST2-RCP, synthesized and accumulated RCNMV CP upon inoculation of *N. benthamiana* (Fig. 3A). RNA-1 was co-inoculated with pHST2-RCP to ascertain whether RNA-1 can be packaged into virions in the absence of RNA-2. The TBSV MP (p22) is able to potentiate the movement of RCNMV RNA-1 for a productive infection. This combination did not produce detectable virions under the EM (Fig. 3B). This lack of virion accumulation, given the necessary components for viral assembly, demonstrates that RCNMV RNA-1 is unable to produce virions in the absence of RNA-2.

To reinforce the requirement of RNA-2 for virion assembly in this assay, we employed an RNA-1 construct that expresses GFP in place of the CP ORF (R1sGFP; Sit et al., 1998). RNA-2 trans-activation of CP synthesis is not an issue since R1sGFP would produce GFP and not CP. The three transcripts, R1sGFP, native RCNMV RNA-2 and pHST2-RCP, were co-inoculated onto *N. benthamiana* and virion formation was assayed by EM. This triple inoculation leads to virion production (Fig. 3B).
This heterologous expression of the RCNMV CP has demonstrated that: i) RNA-1 does not contain a discrete OAS and thus, is unable to form virions by itself and ii) RNA-2 is the packaging determinant for RCNMV virion formation.

A 209 nt region of RCNMV RNA-2 directs encapsidation of heterologous TBSV RNA in the presence of RCNMV CP. Sit et al. (1998) reported that a TBSV construct expressing a 209 nt region from RCNMV RNA-2 directed CP synthesis from RCNMV RNA-1. A new construct containing the same 209 nt region was engineered to ensure that the sgRNA expressed from this TBSV construct is similar in size to wild-type RCNMV RNA-2. This construct, pHST2, was tested for its ability to trans-activate CP expression and form virions when co-inoculated with RCNMV RNA-1. Western analysis revealed that pHST2-ΔBP induces CP expression but at reduced levels in comparison to wild type RNA-2 (Fig. 4A). Virions produced by pHST2-ΔBP co-inoculation were indistinguishable from wild-type virions (Fig. 4B). Assays for virion formation showed accumulation of the chimeric virion to approximately 60% of the wild-type level based on UV spectroscopy of purified virions (data not shown).

Viral RNA extracted from these chimeric virions was subjected to northern analysis with probes corresponding to genomic RCNMV and TBSV RNAs. The RNA-1 specific probe detected the presence of RNA-1 in the chimeric as well as wild-type virions (Fig. 4C). An RNA species was detected in chimeric virions which hybridized to RCNMV RNA-2 as well as TBSV specific probes (Fig. 4C). This RNA species is slightly smaller than RCNMV RNA-2 and appears to be the TBSV sgRNA expressing the 209 nt sequence from RCNMV RNA-2. Furthermore, the TBSV specific probe
did not detect the encapsidation of genomic TBSV RNA despite the presence of the 209 nt sequence.

**Refining the minimal OAS on RCNMV RNA-2 by deletion mutagenesis.**

To refine the packaging signal we created a series of deletion mutants within this 209 sequence (nt 708-917; termed the Origin of Assembly Region or OAR) directly on RNA-2. Five stable stem loop structures were predicted to be present within the OAR utilizing MFOLD (Fig. 5A; Zuker et al., 1999). The first structure is the previously reported TA element (Sit et al., 1998) followed by a GNRA tetraloop with three discrete stem loops (SL1-SL3) further downstream. There are also intervening sequences between these primary structures (g1-g3) which may or may not have functional implications in assembly. The mutants were designed to delete the primary structures and the intervening sequences. Deletion mutants within the OAR, which is located within the MP ORF, may affect MP function. This required transgenic *N. benthamiana* plants that express wild-type RCNMV MP to complement any potential movement defects (Vaewhongs and Lommel, 1995).

Deletion mutants (still containing the TA element) were initially screened for replication by co-inoculation with R1sGFP and assayed for GFP expression. Mutants were subsequently co-infected with wild-type RCNMV RNA-1 and assayed for replication (via northern blotting) and assembly (by EM of purified virions). Deletions of the GNRA tetraloop (R2ΔGNRA) or any of the three downstream stem loops (R2ΔSL1, R2ΔSL2, R2ΔSL3, R2ΔSL1-3) as well as the intervening sequences (R2Δg1 and R2Δg2), were replication competent as assayed by northern analysis (Fig. 5B). However, co-inoculation with R1sGFP demonstrated the replication of
R2Δg3, R2Δg2-g3, R2ΔG-g3 at very low levels (data not shown). Replication was not evident for deletion mutants R2ΔTA and R2Δg1-g3 in either assay.

As a primary screen for virion assembly, deletion mutants were assayed for their ability to induce CP expression (Fig. 5B). Deletion mutants R2ΔTA and R2Δg1-g3 lack the TA element and did not produce CP, as expected. All other deletion mutants, with the exception of R2ΔG-g3 and R2Δg2-g3, induced detectable CP accumulation. Assays for virion production determined that all RNA-2 deletion mutants, again with the exception of R2ΔTA and R2Δg1-g3, assembled into virions with varying degrees of efficiency (Fig. 5C).

In this study, deletion mutants of structural elements within the OAR were engineered to refine the minimal packaging element. Despite deletion of all other structural elements within the OAR, save for the TA element, mutant R2ΔG-g3 was still able to direct virion assembly, although at highly reduced levels. This result indicates that the TA element is the essential structure on RNA-2 that is necessary and sufficient for RCNMV assembly.

**TA loop mutation does not disrupt OAS function.** The terminal loop of the RNA-2 TA element base pairs with the RNA-1 TABS for trans-activation of CP sgRNA synthesis (Fig. 1; Sit et al., 1998). Since the 8 nt terminal loop of the TA element was implicated in trans-activation, we wanted to ascertain if it played an additional role in assembly. To do this, the TA-TABS interaction was disrupted by incorporating two point mutations into the loop (Fig. 6A). This mutant (R2TAMUT) was able to replicate when co-inoculated with either R1sGFP or RNA-1 but did not induce either GFP production from R1sGFP (data not shown) or CP from RNA-1
(Fig. 5B). The assembly capabilities of R2TAMUT were assayed by co-inoculation with RCNMV RNA-1 and pHST2-RCP as the exogenous source of CP. This combination results in the production of virions (Fig. 5C), which contain both RNA-1 and R2TAMUT (Fig. 6B). This suggests that these point mutations in the TA loop have no detrimental effect on assembly.

The RCNMV TA element functions as an OAS in a heterologous context. The TA element was cloned into the heterologous pHST2 vector and was expected to express the TA element within a sgRNA of approximately 1134 nt in length. This construct, pHST2-SLΔNA, was co-inoculated with RCNMV RNA-1 and assayed for packaging. The presence of the TA element in pHST2-SLΔNA results in CP expression from RNA-1 but at a reduced level in comparison to RCNMV RNA-2 (Fig. 7A). Furthermore, virions were observed confirming that the TA element was sufficient for directing virion formation (Fig. 7B). Virion accumulation was less than that produced with RCNMV RNA-2 as expected, based on the western analysis.

RNA extracted from the chimeric virions was analyzed by gel electrophoresis and RT-PCR. As seen in Fig. 7C, chimeric virions contain RNA-1 together with a smaller RNA species corresponding in size to the sgRNA expressed from pHST2-SLΔNA. Viral RNA was further subjected to RT-PCR analysis with TA and pHST2 specific primers to verify the chimeric nature of the packaged RNAs. Fig. 7D shows that the co-packaged sgRNA has retained the TA element. These findings demonstrate conclusively that the TA element functions as the RCNMV OAS.

Encapsidation of heterologous RNAs defines an upper packaging size limit. The RNA content within icosahedral virions is constrained by their spherical
nature. The packaging limits for RCNMV have not been determined. This limit was probed by utilizing pHST2 vector constructs containing the RCNMV OAR (pHST2-ΔBX, -ΔBA and -ΔBP) or the TA element (pHST2-SLΔNA). The lengths of the sgRNAs were varied from 1134-1690 nt to determine the upper packaging limit for RCNMV virions when co-packaged with RCNMV RNA-1. All four constructs induced CP production from RNA-1 (Fig. 8A). Consequently, all four constructs also produced detectable quantities of virions (Fig. 8B). However, the level of virion accumulation varied depending on the size differential from the optimal length of RCNMV RNA-2 despite the shared presence of the OAR. The maximum virion accumulation was observed with pHST2-ΔBP where virion production was ~60% of wild type. The size of sgRNA produced by pHST2-ΔBP is 1430 nt and is closest in size to that of wild-type RCNMV RNA-2. It is not surprising to note that CP accumulation levels mirror virion accumulation levels thus reflecting the stability of CP in the form of virions. pHST2-ΔBX, at 1690 nt, was the least efficient at virion accumulation (~15% of wild type) and may represent the upper RNA packaging limit within RCNMV virions. Conversely, pHST2-SLΔNA, at 1134 nt in length with ~30% packaging efficiency, may hint at a lower packaging limit for RCNMV which was not reached in this study.

**DISCUSSION**

We report here the successful identification of the RCNMV OAS. By characterizing the RNA content within RCNMV virions, we have determined that the
CP sgRNA is excluded from encapsidation into virions. We also demonstrate that RNA-1 does not possess an independent and discrete OAS and is therefore, not packaged in the absence of RNA-2. Furthermore, an upper RNA packaging size limit was approached for RCNMV virions. On RNA-2, we have identified a 209 nt sequence (the OAR) within the MP ORF that initiates assembly with RCNMV RNA-1 when expressed heterologously. Deletion mutagenesis on RNA-2 was used to define the TA element as the essential OAS within the OAR. The TA has previously been characterized to be the trans-activator element directing transcription of CP sgRNA from RNA-1 (Sit et al., 1998) as well as a cis-acting replication element (Tatsuta et al., 2005). Assembly function is a third role for the TA which emerges as a multifunctional and key regulator of RCNMV.

Previous to these studies, the constitution of RCNMV virion populations was not well defined. We have presently demonstrated that RCNMV produces two distinct virion populations, one that co-packages both genomic RNAs and the other that exclusively packages RNA-2 (Chapter 3). Although RCNMV CP sgRNA has previously been observed in infected tissue (Osman and Buck, 1987, 1990), no evidence for sgRNA packaging has ever been reported for the dianthoviruses. While all members of the Tombusviridae family synthesize CP and MP (except dianthoviruses) from sgRNAs, they are not known to encapsidate them (Qu and Morris, 1997). Certain plant viruses, e.g. the Bromoviridae, are known to package their sgRNAs as efficiently as their genomic RNAs (Ahlquist et al., 1981; Brederode et al., 1980; Gould and Symons, 1982). Our results rule out the possibility that RCNMV sgRNA is packaged into virions.
RCNMV virions would appear to have a finite packaging limit for RNA based on the spherical nature of the particles. Co-packaging of various sized TBSV sgRNAs with RCNMV RNA-1 demonstrated an upper size limit for the RNA content within RCNMV virions. That such a limit exists is not surprising given similar findings for a related member of the *Tombusviridae*, TCV (Qu and Morris, 1997). TCV virions did not efficiently package RNAs much larger than the 4051 nt genome with an upper limit of ~4600 nt. Thus, there is likely to be an optimum amount of RNA that can be packaged into an RCNMV virion with some variation above and below this value. Since the wild-type RCNMV genome is 5337 nt (RNA-1 being 3889 and RNA-2 being 1448 nt), we conclude that this is most likely to be the optimal amount of RNA. For our studies, the size of RNA-1 was constant while the size of the co-packaged RNA varied. Not surprisingly, the chimeric TBSV sgRNA which most closely matched wild-type RCNMV RNA-2 in size (pHST2-ΔBP at 1430 nt) was most efficient at virion accumulation. The packaging extremes for the chimeric sgRNAs obtained in this study were 1690 nt (pHST2-ΔBX) and 1134 nt (pHST2-SSLΔNA) which gives an effective RCNMV virion packaging limit of ~5000 – 5800 nt or +/- 400 nt from the optimum. These limits manifested themselves with packaging efficiencies of ~15% at the upper limit and ~30% at the lower limit. It is also a possibility that these RCNMV packaging limits are a result of the instability of chimeric virions and the less than ideal packaging of heterologous RNAs by the RCNMV CP.

Despite the presence of the RCNMV OAS on the genomic pHST2 constructs, TBSV genomic RNA was never encapsidated by RCNMV CP. This is probably due to the stoichiometry of the packaged RNAs where chimeric pHST2 constructs alone
(~4000+ nt) would be insufficient while being too large to co-package with RCNMV RNA-1 (~7900 nt).

With the aid of heterologously expressed RCNMV CP, we were able to demonstrate the absence of a discrete OAS on RCNMV RNA-1. This parallels the situation for another bipartite RNA virus, FHV, where RNA1 does not contain the OAS (Zhong et al., 1992). FHV, like RCNMV, co-packages both genomic RNAs into a single virion (Krishna and Schneemann, 1999). Thus, RCNMV genomic RNAs are truly co-dependent upon each other since RNA-1 cannot package and move without the aid of RNA-2 while RNA-2 cannot replicate without the aid of RNA-1.

Ruling out the presence of a discrete packaging signal on RNA-1, our attention was focused on RNA-2 as the most probable location of the OAS. Sit et al. (1998) determined that a 209 nt sequence from RNA-2 was sufficient to trans-activate CP synthesis from RNA-1. This region (which we now term the OAR) is highly conserved phylogenetically among the three sequenced dianthovirus species possibly reflecting a conserved function. pHST2 constructs containing the OAR co-package RCNMV RNA-1 (Fig. 4B and C), demonstrating the presence of the RCNMV OAS within this region. MFOLD predicted the potential presence of five stem loop secondary structures. This included the structurally characterized TA element that was shown to adopt the proposed structure in solution (Guenther et al., 2004). Given the possibility that any one of these five structures was the putative OAS, what properties do all characterized OASs of spherical viruses have in common?
The best characterized OAS for an icosahedral plant RNA virus is that of TCV which was delimited to a 28 nt bulged hairpin loop (Qu and Morris, 1997). TCV contains a monopartite genome and its packaging is conceptually straightforward. No definitive OAS has been reported for a multipartite plant virus. However, sequence elements responsible for the assembly of BMV RNA3 have been reported. The 3’ TLS in conjunction with a 69 nt stem loop sequence within the MP ORF comprises the bipartite packaging signal (Choi and Rao, 2003; Damayanti et al., 2003). Therefore, the OAS for RCNMV would most likely be a highly conserved stem loop structure.

A series of deletion mutants (Fig. 5A) were constructed on RCNMV RNA-2 in order to find the critical structural element within the OAR responsible for virion assembly. Mutations were produced on RCNMV RNA-2 to ensure that the assembly assay system utilized was comparable to that of wild-type RCNMV. The replication efficiencies of the various deletion mutants were dependent on their position relative to the TA element (Fig. 5A and B). Mutations nearer to the TA element did not replicate as efficiently as mutants located further away. This was not surprising in light of the recent findings of Tatsuta et al. (2005) who demonstrated that the TA element also functions as a cis-acting replication element for RNA-2. This finding was reaffirmed by the observation that mutants R2ΔTA and R2Δg1-g3 (both missing the TA element) did not replicate in the presence of RCNMV RNA-1 (Fig 5B and unpublished data).

All deletion mutants were encapsidated into RCNMV virions with the exception of R2ΔTA and R2Δg1-g3 which were previously shown to be incapable of
replication. Surprisingly, construct R2ΔG-g3, lacking the entire OAR with the exception of the TA element, was packaged into virions in the presence of RCNMV RNA-1. This reductionist approach has revealed the TA element as being the functional entity within the OAR responsible for RCNMV assembly. The highly conserved TA element fits the premise that the RCNMV OAS would be a highly conserved stem loop structure. The obvious test for the TA element being the OAS would be to delete only the TA element from RCNMV RNA-2, thereby abolishing virion formation. However, we cannot uncouple the OAS function of the TA element from its cis-acting role in the replication of RCNMV RNA-2 as observed with deletion mutant R2ΔTA. To bypass this obstacle, the TA element was expressed from the heterologous pHST2 vector (construct pHST2-SLΔNA) in the presence of RCNMV RNA-1. The pHST2-SLΔNA sgRNA containing the TA element was co-packaged with RCNMV RNA-1 into virions proving conclusively that the TA element serves as the RCNMV OAS.

The TA/TABS interaction was initially identified via its role in activating CP synthesis from RNA-1 (Sit et al., 1998). Recent structural studies showed that the TA/TABS interaction forms a stacked helical structure reminiscent of the dimerization signal of HIV-1 (Guenther et al., 2004; Mujeeb et al., 1998). The TA/TABS interaction may also be responsible for the initiation of heterodimer complex formation between RNA-1 and RNA-2 (Chapter 3). This mechanism possibly ensures the co-packaging of both genomic RCNMV RNAs much like the dimerization signal of HIV-1 ensures packaging of two copies of the HIV-1 genomic RNA (Fu et al., 1994; Sakuragi et al., 2003). To evaluate the necessity of this
interaction for RCNMV virion assembly, the TA loop was mutated on RCNMV RNA-2 (mutant R2TAMUT). This RNA-2 mutant formed virions when co-infected with RNA-1 and pHST2-RCP as the heterologous source for RCNMV CP. This appears to suggest that the TA loop is not the critical region of the TA element that confers assembly competency. The alterations to the TA loop, being only two point mutations, may not have sufficiently disrupted the TA/TABS interaction. This may also indicate that CP recognition and assembly initiation occurs at a different region of the TA element.

In Chapter 3, we have shown that some virions contain multiple copies of RNA-2 exclusively. While the TA/TABS interaction potentially enables the co-packaging of RNA-1 and RNA-2 into a single virion, a similar interaction may also be involved in RNA-2 homodimer formation resulting in virions consisting solely of RNA-2. Close inspection of the RCNMV RNA-2 sequence revealed the presence of a six nucleotide sequence (5′-GGGGUU-3′) in the 3′ non-coding region, complementary to the TA loop. Binding of the TA element to this TABS mimic (TABS_M) on a second RNA-2 molecule may emulate the original TA/TABS interaction between RNA-1 and RNA-2. The TA/TABS_M interaction may produce the cognate initiation of assembly structure for CP binding while facilitating the packaging of RNA-2 homodimers into single virions. This conjecture might also explain differences in the viral RNA profiles between wild-type and pHST2-ΔBP chimeric virions. Virion RNA from wild-type infections exhibits an RNA-1: RNA-2 ratio of 1:3 while RNA-1 and pHST2-ΔBP co-infections result in a 1:1 ratio (Fig. 4C). Intriguingly, the TABS_M is absent from pHST2-ΔBP and hence, pHST2-ΔBP sgRNAs would be unable to recreate the
TA/TABS\textsubscript{M} interaction. This would preclude the formation of virions with only pHST2-ΔBP sgRNAs and possibly explain the disparity between the RNA ratios. From these observations and conjecture, we surmise that the RNA-2 only virions are possibly formed as a consequence of the putative TA/TABS\textsubscript{M} interaction that may lead to RNA-2 homodimer formation.

Based on the cumulative data discussed above, we propose a model for RCNMV assembly (Fig. 9). The RNA-2 TA element binds to the RNA-1 TABS and initiates CP sgRNA and subsequently, CP synthesis. The TA/TABS interaction results in a reorganization of the TA element leading to exposure of its stem and followed by CP recognition and binding. The TA/TABS interaction leads to an intimate association between RNA-1 and RNA-2 facilitating encapsidation of both RNAs into a single virion. As a consequence of the TA/TABS\textsubscript{M} interaction between RNA-2 molecules, RNA-2 homodimers may be encapsidated into a separate sub-population of virions. Assuming a finite amount of RNA is required for stable virion formation, this second type of virion would only package two RNA-2 homodimers, giving rise to a virion with four copies of RNA-2. Thus, RCNMV virion preparations are composed of two populations of virions: i) one type co-packaging RNA-1 and RNA-2 (5.3 kb total) and ii) a second type with four copies of RNA-2 (5.9 kb total). This difference in total RNA content per virion may not manifest itself in an observable difference in density on a CsCl gradient, thus fitting the previously observed single band data. Additional support for the model comes from dilution infectivity data for dianthoviruses which suggested two particle types (Hamilton and Tremaine, 1996). Previous analysis of purified RCNMV virion RNA revealed an
RNA-1: RNA-2 molar ratio of approximately 1:3. Based on this observation, the likely ratio for the two virion types within a given preparation is two RNA-1: RNA-2 virions for every one RNA-2-only virion.

The finding in this study that the previously characterized RCNMV RNA-2 TA element also acts as the discrete OAS is not too surprising given the genetic compactness of RNA viral genomes. There are several examples of multifunctional RNA elements within both plant and animal RNA viral genomes. The *Turnip yellow mosaic virus* (TYMV) 5' leader region contains a hairpin secondary structure identified as a regulator of both translation and encapsidation (Bink et al., 2002, 2003; Hellendoorn et al., 1996). This TYMV regulator is involved in translation during the early stages of the viral life cycle. During latter stages of infection, the internal cytosines within the hairpin get protonated leading to an altered conformation and a switch from translation regulator to packaging signal for TYMV encapsidation. The BMV 3' TLS is a multifunctional element that directs minus-strand synthesis (Chapman and Kao, 1999; Dreher, 1999) while also serving as half of the packaging element (Choi et al., 2002; Choi and Rao, 2003; Damayanti et al., 2003).

Genomic functions of HIV-1, such as assembly, dimerization, primer binding, primer initiation and Gag start codon, overlap each other within the 5' non coding region known as the Ψ element (Berkhout, 1996; Clever et al., 2002; Lanchy et al., 2003; Paillart et al., 2004). This Ψ element acts as a riboswitch that upon dimerization of the genomic RNA, changes its conformation to present the essential encapsidation signal to allow the binding of Gag polyprotein (nucleocapsid precursor; Ooms et al., 2004). The dimerization and encapsidation switch
mechanism of the \( \Psi \) element for *Moloney murine leukemia virus* is better characterized (D’Souza and Summers, 2004). Dimerization of the genomic RNA exposes the conserved UCUG sequence to the Gag polyprotein ensuring that only the genomic dimer is packaged while discriminating against spliced RNAs which do not dimerize.

The multifunctional nature of the TA element was reaffirmed by our findings that it functions as the OAS. Much like the retroviruses mentioned previously, the TA/TABS interaction may cause a reorganization of the TA element to expose the critical residues required for CP binding to the OAS found most likely in the TA stem. The TA/TABS interaction may also function to ensure co-packaging of the genomic RNAs into a single virion. One could envision the evolving role of the TA element during the viral life cycle to be a consequence of: i) the RNA-2 concentration, ii) the presence of both *cis*- and *trans*-interactions and iii) the presence of viral proteins. The dynamic TA element would first serve as a *cis*-acting replication element (Tatsuta et al., 2005) at low concentrations of RNA-2 where *trans*-interactions are not favored. Later in the infection process, when sufficient time has elapsed to allow amplification of both genomic RNAs, a *trans*-interaction (TA/TABS) would be the most likely outcome leading to sgRNA synthesis. Finally, this TA/TABS structure may be altered by the process of interaction to reveal the final function of the TA element, that of the OAS. Thus, the TA is a key regulatory element of the RCNMV life cycle.
ACKNOWLEDGEMENTS

Special thanks to Valerie Knowlton for the TEM work. Thanks to Drs. C.L. Hemenway and I.T.D. Petty for useful discussion and guidance. We must thank Dr. E. Gillock for help with western blots. This work was supported by NSF competitive grant MCB-0077964 to S.A.L. and T.L.S.
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</tr>
<tr>
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1Oligonucleotides used with the QuikChange® site-directed mutagenesis kit are denoted with an asterisk. The complementary oligonucleotide is not shown. Engineered restriction sites are shown in parentheses.

2Underscore denotes deleted region. Mutated or inserted nucleotides are indicated in lowercase.
Fig. 1. *Red clover necrotic mosaic virus* genome organization. A schematic representation of the RCNMV genome. The ORFs are depicted as boxes and the encoded products are indicated within the boxes. The protein products, p27 and p88 are replicase proteins, CP is the capsid protein and MP the movement protein. Frame shift position is indicated which is responsible for the translation of p88. The positions and the sequences of the TA and TABS are indicated with complementary regions shaded.
Fig. 2. RCNMV CP sgRNA is not packaged into virions. Northern analysis of viral RNA encapsidated into wild-type RCNMV virions. Purified virion RNA from wild-type infections was subjected northern analysis with $^{32}$P labeled probes corresponding to CP ORF, p27 ORF and RNA-2 respectively. Virion RNA from four samples were analyzed. The probe used is indicated below each blot. The positions of RNA-1, RNA-2 and CP sgRNA are indicated with arrows. Note that no CP sgRNA was packaged.
Fig. 3. RCNMV RNA-1 does not contain a discrete OAS. RNA-1 is not packaged in the absence of RNA-2. A. A western blot of RCNMV CP expressed from the TBSV vector. B. Electron microscopic images of RCNMV virions. Virions purified from co-inoculations were observed under a transmission electron microscope. A schematic representation of the constructs are shown in left. The presence (+) or absence of virions (-) is denoted. Scale bar equals 50 nm.
Fig. 4. RCNMV virion assembly directed by the TBSV sgRNA containing the RCNMV RNA-2 OAR fragment. A. CP expression induced by RNA-2 OAR. A western blot of the RCNMV CP expressed by co-inoculations with the respective constructs are shown. RNA-1 was co-infected with pHST2-ΔBP and RNA-2 respectively, CP extracted and analyzed by western blotting. B. Electron microscopic images of RCNMV virions formed. A schematic representation of the constructs used in the experiment are indicated to the left of each image and presence (+) or absence (-) of virions are indicated. C. Determining the packaged RNA within chimeric virions. The RNA-2 OAR element expressed from the TBSV vector forms virions when co-inoculated with RNA-1 and the virion RNA is analyzed by northern blotting. Probes corresponding to RNA-1, RNA-2 and TBSV genomic RNA were used. Note that the packaged TBSV sgRNA contains the RNA-2 OAR element and smaller than native RNA-2. Encapsidated RNA species are denoted with asterisks (*-RNA-1, **- RNA-2, ***-TBSV sgRNA).
A.

\[
\begin{align*}
\text{R2}\Delta g1 & (1405) & \text{R2}\Delta TA & (1408) & \text{R2}\Delta GNRA & (1427) \\
\text{R2}\Delta g2 & (1427) & \text{R2}\Delta SL1 & (1426) & \text{R2}\Delta SL2 & (1426) \\
\text{R2}\Delta SL3 & (1431) & \text{R2}\Delta g3 & (1427) & \text{R2}\Delta SL1-3 & (1378) \\
\text{R2}\Delta g2-g3 & (1359) & \text{R2}\Delta G-g3 & (1329) & \text{R2}\Delta g1-g3 & (1263) \\
\text{R2TAMUT} & (1448)
\end{align*}
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**B.**

RNA-2

rRNA

CP
C.

R2ΔSL1  R2ΔSL2  R2ΔSL3

R2ΔSL1-3  R2ΔTA  R2ΔGNRA

R2Δg1-g3  R2TAMUT  R2Δg1

R2Δg2  R2Δg3  R2Δg2-g3

R2ΔG-g3  RNA-2 (wt)

50 nm
Fig. 5. Virion formation of the RNA-2 deletion mutants. A. The secondary structure prediction (Mfold) for RNA-2 OAR element (nts 708-917). The putative five stem loop structures (TA, GNRA, SL1, SL2 and SL3) and the intervening sequences (g1, g2 and g3) are indicated. The nucleotide positions of the deletions are given. The TA and GNRA loops are shaded. A schematic representation of the RNA-2 deletion mutants are given below. The OAR element is represented by the black line with the positions of the putative secondary structures indicated. Black lines with the deletion signs indicate the deleted regions. Size is indicated next to each construct. The ‘*’ indicates the position of the nucleotide mutation on R2TAMUT. B. Replication and CP expression of the RNA-2 deletion mutants. A northern analysis of total RNA isolated from virions produced by co-inoculations of the deletion mutants with RNA-1. The blots were hybridized to ³²P labeled RCNMV RNA-2 specific probes. The panel below represent the rRNA control from each total RNA extraction. Western blotting of the infections were prepared to determine the CP accumulation in each co-inoculation. C. Packaging efficiency of the RNA-2 deletion mutants. RNA-2 mutants were co-inoculated with RNA-1 (in the R2TAMUT inoculation, pHST2-RCP was also co-inoculated to provide CP to the infection) and virions were visualized under TEM. The scale bar is 50 nm.
Fig. 6. The role of TA terminal loop in assembly. A. Base pairing of the TA and TABS sequences. The wild-type TA-TABS interaction is depicted in the left; right, two nucleotides of the TA terminal loop were mutated and its effect on virion formation was determined. B. Northern hybridization analysis of virion RNA produced by RNA-1 and R2TAMUT co-inoculations. Since R2TAMUT does not induce CP expression from RNA-1, pHST2-RCP was co-inoculated to provide CP.
Fig. 7. The RCNMV RNA-2 TA is the OAS. A. A western blot of CP induced by the TBSV vector expressing RNA-2 TA(pHST2-SL ΔNA) when co-inoculated with RNA-1. B. Electron micrographic images of virions from RNA-2 and pHST2-SLΔNA when co-inoculated with RNA-1. The constructs used in the inoculations are given in the left. C. Encapsidated viral RNA. Purified virion RNA from RCNMV virions was analyzed by gel electrophoresis. The arrows denote the size of the smaller RNA species. Asterisks denote the RNA species packaged into RCNMV virions: *-RNA-1 (3889 kb), **-RNA-2 (1448 kb), ***- TBSV sgRNA (pHST2-SLΔNA, 1134 b; pHST2-ΔBP, 1430 b). D. RT-PCR amplification of virion RNA to verify the encapsidation of pHST2-SLΔNA sgRNA. RT-PCR was conducted with primers specific to both TA and pHST2. Virion RNA from the RNA-2 positive control, was amplified with RNA-2 specific primers. Note that the amplified products from pHST2-SLΔNA and pHST2-ΔBP are different in size. This reflects the sizes of the sgRNA produced by each construct.
A.

RNA-1

RNA-2

RNA-2

B.

RNA-1

RNA-2

RNA-2

RNA-2

RNA-2

RCNMV CP

100 %

1448

15 %

1690

60 %

1430

35 %

1290

30 %

1134

50 nm
Fig. 8. RNA size requirement for RCNMV packaging. A. Western blotting of CP synthesized by respective pHST2 vectors containing the RNA-2 sequences. RNA-1 was co-inoculated in each of the infections. The pHST2 constructs containing the RNA-2 sequence was engineered to produce different sized sgRNAs. The pHST2-ΔBX, pHST2-ΔBP, pHST2-ΔBA constructs contained the RNA-2 OAR fragment whereas pHST2-SLΔNA carried the TA sequence. B. Virion production by the pHST2 constructs. Electron micrograph images of the respective inoculations and the constructs used are depicted in the left. Presence (+) or absence of virions (-) is denoted as well as the level of virion accumulation of each of the combinations. The sizes of the sgRNA produced by each construct is indicated below each sgRNA.
Fig. 9. An RCNMV assembly model. A schematic representation of the RCNMV RNA-1 and RNA-2 interacting with each other. The CP subunits recognize the packaging signal on RNA-2, initiating assembly to produce virions. RNA-1, RNA-2 or RNA-2 dimerization may expose the packaging signal to CP. The resultant virions could contain either one copy each of RNA-1 and RNA-2 or multiple copies of RNA-2. The TA, TABS and TABSn are indicated.
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Chapter 5

*Red Clover Necrotic Mosaic Virus* Particles Consist of Both Native and Expanded Forms

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Abstract

Red clover necrotic mosaic virus (RCNMV) particles are composed of 37 kDa capsid protein (CP) subunits arranged with T=3 icosahedral symmetry encapsidating the two genomic ssRNAs of 3.9 and 1.5 kb. RCNMV virions range in size from 30-35 nm in diameter. Previous work in our laboratory demonstrated the heterogeneity of RCNMV virions, where one virion co-packages the two genomic RNAs and the other packages possibly four copies of RNA-2 (Chapter 3). The RCNMV virion population when subjected to density gradient centrifugation has displayed a singular density. To further purify and identify any subtle differences in density within the RCNMV virion component, they were subjected to centrifugal separation utilizing an iodinated medium (Optiprep™). Centrifugation of RCNMV virions in the iodinated medium resulted in the separation of the RCNMV population into two fractions. We demonstrate that RCNMV virions are composed of both native and expanded forms and that the native fraction is approximately 70% of the total population. Both the native and expanded virions comprise of unmodified CP and encapsidate similar genomic RNA complements. This study demonstrates that an RCNMV virion population purified from a systemic host consists of a combination of virions possibly representing successive stages in the viral life cycle.
Introduction

Virus assembly resulting in a stable virion is achieved by extensive RNA-protein and protein-protein interactions (Ahlquist et al., 1994). Virion capsids undergo major structural changes in assembly and disassembly processes and identifying and characterizing these structural reorganizations are essential in understanding the viral life cycle.

The morphology and stability of plant viruses are affected by pH, temperature and ionic strength (Johnson and Speir, 1997; Speir et al., 1995). Native virions, mostly stable at low pH, are known to undergo radial expansion by 10% upon raising pH under low ionic strength. This phenomenon also termed swelling is observed in a multitude of plant viruses such as Tomato bushy stunt virus (TBSV), Turnip crinkle virus (TCV), Cowpea chlorotic mosaic virus (CCMV) and sobemoviruses (Hsu et al., 1976; Robinson and Harrison, 1982; Sorger et al., 1986; Speir et al., 1995). The expansion of the virions is suggested to be as a consequence of the electrostatic repulsion at the quasi-threefold axes from the loss of protein-binding cations or protons. Acidic residues that bind calcium cations are compactly condensed and upon the loss of neutralization of charge are positioned too close to each other to maintain the original structure. Crystallographic and cryo-EM studies of swollen virions indicate that virion expansion is mainly due to large structural changes in the trimer interface while the hexameric and pentameric plates are largely intact (Robinson and Harrison, 1982; Speir et al., 1995). The swelling of virions while conserving the hexamer and pentamer structural integrity is involved in incremental rotations that allow the expansion at the quasi-threefold axis (Liu et al., 2003; Wang
et al., 2001). Swelling of virion particles, a common occurrence among many viruses has been suggested to reflect an intermediate in disassembly and infection process (Brisco et al., 1986).

RCNMV, a member of genus *Dianthovirus*, family *Tombusviridae*, is a small icosahedral virus with an ssRNA genome of RNA-1 (3.9 kb) and RNA-2 (1.5 kb). RNA-1 encodes p27, replicase-related protein, p88, RNA-dependent RNA polymerase and CP (Xiong et al., 1993b; Xiong and Lommel, 1989) while RNA-2 codes for the movement protein Lommel et al., 1988; Osman et al., 1991; Xiong et al., 1993a). The RCNMV virion is made up of 180 copies of the 37 kDa CP subunit arranged with a T=3 quasi symmetry (Lommel et al., 1988; Xiong and Lommel, 1989). We recently identified the packaging scheme of RCNMV virions and of the two types of virions formed, one co-packages RNA-1 and RNA-2 while the other packages possibly four copies of RNA-2 (Chapter 3). Although the cryo-EM structure of RCNMV has not been completely solved, it has been determined that the outer virion shell shares high similarity with TBSV, a member of *Tombusviridae* family (R.H. Guenther, personal communication). The inner shell of RCNMV virion was resolved to form a highly ordered hexagon composed of RNA in association with the β-annuli of CP and it is hypothesized that RCNMV virion organization, internal and external, closely resembles that of TBSV. As with any other virus, the stability of RCNMV is affected by external physical conditions although to a lesser degree than the other dianthoviruses (Hamilton and Tremaine, 1996). Dianthovirus particles swell when pH is changed from 5.0 to 7.5 (Kuhne and Eisbein, 1983) and most virions are swollen after 24 hr at pH 7.5. EDTA treatment was shown to increase rate of
swelling while the addition of Mg$^{2+}$ or Ca$^{2+}$ cations prevent it (Hamilton and Tremaine, 1996; R.H. Guenther, personal communication). Since swelling of virions has been attributed to large reorganizations within the virion shell and suggested as either a transitional stage of disassembly (Brisco et al., 1986) or an intermediate in assembly (Opalka et al., 2000), it is relevant to identify similar structural alterations in the RCNMV virion shell in order to better characterize its assembly and/or disassembly processes.

Centrifugation has been an important method of purification and analysis of viruses. To analyze and process viruses it is imperative that they are removed from the host cellular material. Although various purification methods have been utilized for viruses over time, most are known to cause both physical and biochemical injury. The effects of gradient media on viruses have been extensively studied (Vanden Berghe, 1983). Sucrose and cesium chloride (CsCl) are two media commonly utilized in virus purification procedures while iodinated media are presently emerging as alternatives. While each virus had a specific reaction to the gradient medium used, the non-ionic iodinated media and sucrose were found to cause the least loss of infectivity. CsCl, a common density gradient medium, was found to cause a significant loss of infectivity to a variety of viruses, as a consequence of its ionic nature (Anderson et al., 1995; Buck et al., 2004; Vanden Berghe, 1983). The iodinated medium was physically gentler on viruses and improved infectious virion yields (Zolotukhin et al., 1999; Dettenhofer and Yu, 1999). Sucrose and CsCl were found to be toxic to host cells and were needed to be removed by dialysis prior to infectivity studies however viruses purified in iodinated media could be analyzed.
directly in animal or plant host systems. Sucrose and CsCl gradients are vastly hyperosmotic and sucrose is also highly viscous and therefore difficult to be removed from the virus preparation. Iodinated media are iso-osmotic and have a lower viscosity. Viruses exhibit lower densities (1.18-1.22 g/ml) in iodinated media (Moller-Larsen and Christensen, 1998) whereas they have densities up to 1.26 g/ml in sucrose and 1.34 g/ml in CsCl respectively. Iodinated media were also especially useful for the purification of plant viruses that have a tendency to aggregate or are unstable in CsCl (Gugerli, 1984). Iodinated media are derivatives of triiodobenzoic acid and are capable of banding any biological molecule or complex, according to its buoyant density, under iso-osmotic conditions (Axis-Shield Applications; URL, http://www.axis-shield-poc.com/images/appl_prod_2003.pdf). Iodixanol is a non-ionic iodinated compound and given the name 5,5 –[(2-hydroxy-1,3-propanediyl)-bis (acetylimino)]bis-[N,N bis(2,3 dihydroxypropyl)-2,4,6-triiodo-benzenedicarboxamide]. Optiprep™ is the trademark name for a sterile 60% (w/v) solution of iodixanol in water. The high density of the solution is due to the substituted triiodobenzene rings with links to a number of hydrophilic groups.

Isopycnic gradient centrifugations are used to separate particles according to their buoyant or banding density. Centrifugation, carried out for an extended period, results in the particles banding at an equivalent density, irrespective of the size or the starting position.

RCNMV virions have previously been observed to band as a single entity in CsCl buoyant density gradients with a density of approximately 1.363 g/cm³ (Hollings and Stone, 1977). Optiprep™ iodinated medium was utilized to improve purification
and separation of RCNMV virions. We report here the separation and identification of native and expanded (swollen) forms of RCNMV virions by density centrifugation using the iodinated medium Optiprep™. Approximately 70% of the virion population consisted of the native form with a diameter of ~30 nm while the rest composed of swollen virions with a diameter of ~35 nm. Analysis of encapsidated virion RNA revealed that both native and swollen virions packaged the same complement of genomic RNAs and in identical molar ratios. Protein analysis of the virion CP did not display modifications. Based on our observations, we propose that the expanded form of RCNMV virion may be an intermediate in the \textit{in vivo} assembly of virions.

\textbf{Materials and methods}

\textbf{Plant inoculation and virion extraction}

RCNMV RNA-1 and RNA-2 were transcribed as previously described (Xiong and Lommel, 1991). Transcripts (5 µl each) in 110 µl 10 mM sodium phosphate buffer, pH 7.0 were used to inoculate four carborundum-dusted leaves of \textit{Nicotiana benthamiana} and/or \textit{N. clevelandii} at the six- to eight-leaf stage. The inoculated plants were grown at 18-20°C under standard glasshouse conditions. RCNMV virions were extracted from infected leaf tissue 4 days post inoculation (d.p.i.) according to the following protocol. Leaf tissue (0.5 g) was homogenized in 1 ml 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 separate cell debris. The supernatant was filtered through miracloth and virions were precipitated by the addition of ¼ volume
40% PEG 8000 in 1 M NaCl on ice. The virions were pelleted by centrifuging at 16,250 \( g \) for 10 minutes and resuspended in 50 \( \mu l \) 10 mM Tris-HCl, pH 6.5.

**Electron microscopy**

Formvar-coated copper grids were layered with the purified virions, stained with 2% uranyl acetate and visualized with a JEM-100 S transmission electron microscope at 50,000 X magnification.

**Virion RNA purification and gel electrophoresis**

Viral RNA was extracted from virions with phenol-chloroform after addition of SDS to 2% final concentration (Lommel et al, 1988). RNA in the aqueous phase was precipitated by the addition of 1/10 volume of 3 M sodium acetate, pH 4.8 and 2.5 volumes of 95% ethanol. The virion RNA was pelleted by centrifugation (13,500 \( g \) for 5 min) and resuspended in 10 \( \mu l \) of nuclease-free water. A 1 \( \mu l \) aliquot of the extracted RNA from was electrophoresed on a 1%-agarose gel in Tris-borate-EDTA (TBE) buffer at 90 V for 1 hour and visualized with ethidium bromide staining.

**Optiprep isopycnic gradient purification**

Optiprep™ (Axis-Shield) solutions were adjusted to 10 mM Tris-HCl, pH 6.5 and discontinued Optiprep™ gradients of 30-40-5-60% were synthesized by overlaying at room temperature. Purified RCNMV virion aliquots (250-500 \( \mu g \)) in 200 \( \mu l \) of 10 mM Tris-HCl, pH 6.5 was layered on top of the gradient. Polyallomer bell-top quick seal tubes (Beckman) were used in centrifugation. Tubes were then
centrifuged in a fixed angle rotor TLA 100.3 (Beckman) at 74,100 g for 16 hours at 5°C. After centrifugation the light scattering virus band was visualized in a dark room under oblique illumination of the tube. The virion bands were collected by puncturing the tubes below the bands. The collected fractions were diluted 5-fold in 10 mM Tris-HCl, pH 6.5 and centrifuged at 296,600 g for 24 min at 5°C. The virion pellet was resuspended in 100 µl of the 10 mM Tris-HCl, pH 6.5. CsCl isopycnic gradient centrifugation was conducted in a similar manner.

**Western blotting**

The RCNMV virions separated using Optiprep™ density gradients and resuspended in Tris buffer were gel electrophoresed. Virion samples, 6 and 2 µl each respectively from the top and bottom components, as well as a combination of 1 and 4 µl of top and bottom fractions were analyzed. The samples were applied to 12% pre-cast acrylamide SDS-PAGE gels (BioRad) and separated at 200 V for 50 min in Laemmli running buffer (Laemmli, 1970) in a BioRad Protean II apparatus. The proteins were transferred to nitrocellulose membranes (GE Osmonics) in transfer buffer (25 mM Tris, 40 mM glycine) for 35 min at 15 V utilizing a BioRad SD cell transblotter. The membrane was incubated in 3% Carnation low fat dry milk in TBST buffer (50 mM Tris, 0.15 M NaCl, 0.05% Tween-20, pH 7.4) overnight. RCNMV CP expression was detected by incubating with rabbit polyclonal antiserum raised against RCNMV CP, followed by horseradish peroxidase linked anti-antibody (Promega). The proteins were visualized with the ECL (Amersham) western blot.
detection kit as directed by the manufacturer and exposed to Kodak Chemiluminescence MS film.

Results

Optiprep™ and CsCl density gradient separation yields swollen and native forms of RCNMV virions

RCNMV virions purified from infected *N. benthamiana* appeared cloudy and milky white in appearance. Approximately 250-500 µg of the virus sample was layered atop the Optiprep™ gradient and centrifuged in a fixed angle rotor. This was to generate an isopycnic gradient in order to separate virions of similar density. Centrifuged virion sample was observed by oblique illumination and the separation resulted in the appearance of two distinct bands of RCNMV virions (Fig. 1 A). The upper fraction banded in the 40% density range and the lower band in the 50% range within the 30-60% Optiprep™ gradient. The upper band was diffused and appeared to be cloudy whereas the lower band was very distinct and sharp. A similar banding pattern was also observed when CsCl was used as the density gradient medium. Virions isolated from the two fractions and from both CsCl and Optiprep™, when observed under the transmission electron microscope, displayed different morphological characteristics. Virions obtained from the upper fraction were spherical and approximately 35 nm in diameter and thus based on their size we can infer that they were swollen (Fig. 1 B). The virions that banded in the lower were smaller (~30 nm in diameter) with angular margins, compact and hexagonal in
appearance and possibly are the native forms. Furthermore, a majority of the swollen virions were filled with stain as further proof of their expansion. The top fraction also contained protein aggregates as well as dissociated particles. Approximately 70% of the RCNMV virion population separated by Optiprep™ consisted of the native virions. Based on these observations, we conclude that the use of Optiprep™ can be successfully used to separate swollen RCNMV virions from the native virions.

**Differential density of the RCNMV virions is not due to a modification of CP**

Virus CP subunits are modified with sugar or lipid moieties leads to a differential range in density for CP subunits (Casaday et al., 2004; Fernandez-Fernandez et al., 2002; McNabb and Courtney, 1992). Modification of proteins leads to a subtle increase in the molecular weight of the protein. This increase in molecular weight can be easily determined by gel electrophoresis of the modified proteins when run alongside unmodified proteins due to their differential mobilities. The virion fractions separated by CsCl and Optiprep™ were electrophoresed on a SDS-PAGE gel. The 37 kDa CP band was identified in both fractions (Fig. 2) and no changes in CP mobility were observed. A smaller band ~25 kDa, corresponding to a degraded CP product was also observed in the two forms of virions. These results suggest that the RCNMV CP subunits of either swollen or native virions were not differentially modified with respect to each other.
Virion RNA composition of the swollen and native RCNMV virions

To determine the RNA complement within the swollen and native virions, virion RNA were analyzed by gel electrophoresis. This was in order to ascertain if the change in density and diameter of the two forms of RCNMV virions was due to packaging of additional RNA. Both the swollen and native virions packaged RNA-1 and RNA-2 and exhibited similar molar ratios for the two RNAs (Fig 3). No auxiliary RNA species were observed in the RCNMV virions and therefore change in density observed for the two virions does not appear to differentially package RNA.

Discussion

Dianthoviruses are a group of small spherical viruses, encapsidated in T=3 quasi-symmetry virion particles made up of chemically identical 180 CP subunits. RCNMV virions were previously demonstrated to be of single density with a diameter of 30-35 nm. The iodinated density gradient medium, Optiprep™, was used as a purification medium and here we report the successful use of the iodinated medium to separate RCNMV swollen virions from the native forms. RCNMV virions purified by Optiprep™ did not exhibit significant disruptions. Based on the results it appears that wild type RCNMV virions, consist primarily (~70%) of native forms in combination with swollen virions. The top fraction consisting of swollen virions also contained dissociated virions and virus debris. This may explain the wider band observed upon fractionation. This is not the first report of the observation of a less dense fraction of RCNMV virions observed from density gradient centrifugation. The
Swedish strain of RCNMV was also found to contain an additional less dense component at 1.356 g/ml (Hollings and Stone, 1977). When CsCl was used as the density gradient medium, a clear spatial separation of the two bands was not observed whereas Optiprep™ gradient centrifugation resulted in a distinct separation of the two fractions. Our results suggest that Optiprep™ is a highly suitable medium for the purification of RCNMV virions. The swollen virions exhibited a ~16% radial expansion and was round in appearance. The virions isolated from the bottom fraction were angular, polyhedral in appearance and were more compact. They are likely the native form of RCNMV virions since a higher proportion of the virions exhibited this compact morphology and size. The encapsidated RNA and the ratios of the RNA complement are identical in swollen and native virions, and it is likely that the variance in density is due to the change in volume of the virions as a consequence of expansion.

Swollen virions displayed a differential staining property where they were filled with uranyl acetate. This may have been due to the expansion of the cavities between the quasi-threefold axes enabling the passage of stain. Formation of large holes in the quasi-threefold axes of swollen capsid shells has previously been reported for CCMV (Speir et al., 1995).

CsCl is known to disrupt virions (Gugerli, 1984) that may result in their swelling. Since we observed swelling of RCNMV virions upon Optiprep™ separation, which is iso-osmotic and been demonstrated to be less disruptive, it is likely that the swollen virions were present in the intracellular environment prior to virion extraction and that are not an artifact of the purification procedure.
CP-CP interactions in a virus shell are known to provide stability to the virion structure (Ahlquist et al., 1994). Paradoxically, a virion must be stable enough to protect the viral RNA from the exterior and yet capable of disassembly once within the cell. Virions are believed to undergo structural transitions within the cell to allow for the release of genomic RNA. A model for the disassembly of CCMV has been proposed where the structural reorganization of the capsid shell grants access to the host ribosomes for translation which results in stripping of the genomic RNA by the ribosomes (Brisco et al., 1986). This model termed cotranslational disassembly is proposed to occur subsequent to swelling of the virions. Recent work on CCMV however, disputed this observation where swelling was not required for disassembly. Instead a structural reorganization of the hexameric tubular structure was demonstrated to present the viral RNA through the virion fivefold axis (Albert et al., 1997). A similar mode of delivery for viral RNA was also proposed for the insect-infecting, bipartite and spherical Flock house virus (Cheng et al., 1994).

Round, expanded forms of virions have been identified as virion precursors that upon maturation forms biologically functional and compact polyhedral forms (Canady et al., 2000). The fact that many viral cores [hepatitis (Bottcher et al., 1997; Conway et al., 1997), Sindbis (Paredes et al., 1993)] are round lends support to this view. Since these cores are protected by an outer layer they are thus optimized for ease of assembly rather than structural integrity. Since non-enveloped virions do not contain the outer membranous covering, in order to protect their genomic RNA, they may utilize mechanisms such as compact packing to produce more stable virions. The sobemovirus Rice yellow mottle virus (RYMV), a non-enveloped virus, was
proposed to produce expanded and rounded virions as intermediates in \textit{in vivo} assembly that on phloem translocation (as a result of the displacement of Ca$^{2+}$ cations from the pit membranes), forms compact virions with closer packing to generate stable virions (Opalka et al., 2000). These observations suggest that the swollen virions identified from many viruses, is a transitional form of assembly rather than of disassembly. The poliovirus capsid shell on the other hand is known to expand during receptor attachment which reflecting a stage prior to disassembly (Fricks and Hogle, 1990).

Although swelling prior to treatment has not been previously identified with dianthoviruses, swelling as a consequence of change in pH or stripping of cations by EDTA has been reported (Hamilton and Tremaine, 1996; Tremaine and Ronald, 1976).

TBSV and TCV (members of the \textit{Tombusviridae} family) exhibit swelling when stripped of cations or when purified in the absence of cations. A possible explanation is that the rounded and expanded forms occur as intermediates in assembly. RCNMV virions isolated from a plant host 4 d.p.i. inoculation likely contain a virion population in an advanced stage of maturity. Since a high proportion of the virions (~70%) were in the compact form, we propose that the swollen virions are pre-assembly intermediates that form compactly folded stable virions possibly prior to vascular transport. This would ensure that the virions are in a stable, tightly packed form to prevent degradation of the genomic RNA upon passage through the vasculature. Furthermore, RCNMV has been hypothesized to require virion formation for systemic spread (Vaewhongs and Lommel, 1995). Virion expansion
possibly occurs as a consequence of an increase in the ionic concentration within the cell that results in an influx of cations, leading to the compact folding of the virion. There may be extensive structural reorganizations of the capsid shell that result in the formation of these compact virions.

The swelling of RCNMV virions occurred in the absence of a change in pH or cationic concentration, however, it is likely that a loss of cations occurred during the purification procedure that may have resulted in swelling. Reversible swelling of RCNMV virions has been observed upon EDTA treatment (R.H. Guenther, personal communication). The cation stripping may likely revert the virions back to pre-assembly expanded forms. Therefore, it is likely that the swollen RCNMV virions would form compactly folded stable virions upon being supplemented with cations. Cryo-EM reconstructions of the EDTA-treated and swollen RCNMV virions exhibited drastic changes in the organization of the interior virion shell and these structural changes of the shell were observed to expose the encapsidated RNA to the exterior (R.H. Guenther, unpublished data). When RCNMV virions were treated with EGTA (to selectively remove CA$^{2+}$ cations), it did not result in an obvious structural change but some reorganization of the interior shell was observed. This suggests that Mg$^{2+}$ is the major cation determinant of RCNMV capsid shell structural transition. Interestingly, either EGTA or EDTA did not result in an obvious expansion of the virions.

The range in diameter observed for RCNMV virions is 30-35 nm and the native and swollen forms of RCNMV virions clearly reflect this range.
Fig. 1. Separation of swollen and native RCNMV virions. A. Optiprep™ fractionation of RCNMV virions. Purified RCNMV virions were fractionated in an Optiprep™ density gradient of 30-60%. The top and bottom fractions are indicated by arrows. B. Electron micrographic images of RCNMV virions fractionated in both CsCl and Optiprep™ media. The diameter of the top (swollen) and bottom (native) virions are shown.
Fig. 2. CP of swollen virions do not appear to be modified. RCNMV virions were fractionated by Optiprep™ and western blotting was conducted. The molecular weight of CP (37 kDa) is indicated. The 25 kDa is a degraded product of CP.
Fig. 3. RCNMV viral RNA purified from native and swollen virions. Virion RNA was purified from the two types of virions and analyzed by gel electrophoresis. The molecular weights of RNA-1 (3.9 kb) and RNA-2 (1.5 kb) are indicated.
References


