Intraspecies Host Specificity of a Single-Stranded RNA Virus Infecting a Marine Photosynthetic Protist Is Determined at the Early Steps of Infection

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Viruses are extremely abundant in seawater, and are believed to be significant pathogens to photosynthetic protists (microalgae). Recently several novel RNA viruses were found to infect marine photosynthetic protists, one of them is HcRNAV which infects *Heterocapsa circularisquama* (Dinophyceae). There are two distinct ecotypes of HcRNAV with complementary intraspecies host ranges. Nucleotide sequence comparison between them revealed remarkable differences in the coat protein coding gene resulting in a high frequency of amino acid substitutions. However, the detailed mechanism supporting the intraspecies host specificity is still unknown. In this study, virus inoculation experiments were conducted with compatible and incompatible host/virus combinations to investigate the mechanism determining the intraspecies host specificity. Cells were infected by adding a virus suspension directly to a host culture or by transfecting viral RNA into host cells using particle bombardment. Virus propagation was monitored by Northern blot analysis using a negative-strand-specific RNA probe, transmission electron microscopy, and a cell lysis assay. Using compatible host/virus combinations, propagation of infectious progeny occurred regardless of the inoculation method. When incompatible combinations were used, direct addition of virus suspension did not even result in viral RNA replication; while, in the host cells transfected with viral RNA, infective progeny virus particles with a host range encoded by the imported viral RNA were propagated. This indicates intraspecies host specificity of HcRNAV is determined by the upstream events of virus infection. This is the first report describing the reproductive steps of an RNA virus infecting a photosynthetic
protist at the molecular level.

INTRODUCTION

Viruses are extremely abundant in seawater (29) and are considered to play a major role as pathogens of marine primary producers photosynthetic protists (microalgae). Algal viruses were previously assumed to consist almost entirely of double-stranded DNA viruses that belong to the family Phycodnaviridae (28). However, recent investigations revealed previously unknown RNA viruses also infect marine photosynthetic protists. HaRNA V (15, 30) and RsRNA V (25) are positive-sense single-stranded RNA viruses that infect toxic-bloom forming microalga Heterosigma akasiwo (Raphidophyceae) and diatom Rhizosolenia setigera (Bacillariophyceae), respectively. Although the genomes of HaRNA V and RsRNA V have some characteristics similar to viruses in the proposed order Picornavirales, phylogenetic analysis based on the deduced amino acid sequence of the RNA-dependent RNA polymerase (RdRP) domain suggest that these two viruses have an independent lineage that evolved from a deep internal branch in Picornavirales (6). MpRNA V is a double-stranded RNA virus in the family Reoviridae that infects the cosmopolitan photosynthetic protist Micromonas pusilla (Prasinophyceae)(1).

HcRNA V infects the bloom-forming photosynthetic dinoflagellate Heterocapsa circularisquama (32). The genome of HcRNA V consists of 4.4 kb of undivided positive-sense single-stranded RNA with no cap structure at the 5'-end (H. Mizumoto...
3'-terminal sequence of HcRNAV is predicted to construct a stable stem-loop structure (20) as observed in other non-polyadenylated RNA viruses (19). The genome of HcRNAV contains two open reading frames (ORFs) (20). The deduced amino acid sequence suggests ORF-1 contains two conserved domains coding for essential replication enzymes: a serine protease domain and an RdRP domain (20). This implies ORF-1 codes for a 110-kDa polyprotein that is translated and cleaved into smaller functional proteins including the RdRP. Phylogenetic analysis of the RdRP amino acid sequence suggests HcRNAV belongs to a previously unrecognized virus group (20).

ORF-2 codes for a 38-kDa viral coat protein (CP) (20).

Previously we showed that HcRNAV infection is ecotype-specific rather than species-specific. Using a cross-reactivity test with 56 clonal H. circularisquama isolates and 107 clonal virus isolates, we found HcRNAV is divided into two distinct ecotypes having complementary intraspecies host specificity: ecotypes UA and CY (32). Analyses of representative virus isolates belonging to each ecotype (HcRNAV34 and HcRNAV109; UA and CY, respectively) show the viral genomes are about 97% identical to each other at nucleotide sequence level (Fig. 1) (20, 32). The noticeable difference between these two viral genomes is four variable regions (I – IV) in ORF-2 with a high frequency of amino acid substitutions (Fig. 1). We assume this determines the intraspecies host specificity of HcRNAV; i.e., viral cell tropism (20); However, the detailed mechanism supporting cell tropism for HcRNAV is not fully understood.

The major step determining tropism is the specific attachment of viruses to
susceptible host cells, i.e., viral tropism can be determined by the cellular receptor-viral ligand interactions. The most intensively studied example is poliovirus. The immunoglobulin super-family molecule CD155 is regarded as the sole human cellular binding molecule conferring susceptibility to poliovirus (17). Although rodents ordinarily resist poliovirus infection due to the absence of CD155, transgenic mice expressing CD155 became susceptible to poliovirus infection (23). Another determinant of tropism is virus replication. Poxvirus tropism is considered to be regulated by intracellular events downstream of virus binding and entry, rather than at the level of specific host receptors (16).

The objective of this study is to determine whether the intraspecies host specificity of HcRNA V is determined at the entry process or the intracellular multiplication process. Instead of using the conventional virus-induced cell lysis assay, we examined viral RNA accumulation using a highly sensitive strand-specific Northern blot analysis. In addition, a direct transfection of viral RNA into host cells was performed to analyze viral RNA replication. Our results show intraspecies host specificity of HcRNA V is determined at early steps of virus infection. This is the first report showing the reproduction steps of RNA viruses infecting photosynthetic protists at the molecular level.

**MATERIALS AND METHODS**

**Hosts and viruses**

The origin of the virus isolates (HcRNAV34 and HcRNAV109) and host
photosynthetic protist isolates (Heterocapsa circularisquama HU9433-P and HCLG-1) used in this study were previously reported (32). Cell cultures were grown in modified SWM3 medium enriched with 2 nM Na$_2$SeO$_3$ (4, 11, 13) and incubated under a 12 h light: 12 h dark cycle; the light (130 to 150 µmol photons m$^{-2}$ s$^{-1}$) was provided by cool white fluorescent illumination at 20°C. Because HcRNA isolates have an ecotype-specific host range (32), HcRNAV34 and HcRNAV109 were maintained using their suitable hosts, HU9433-P and HCLG-1, respectively. The virus stock was inoculated into a fresh culture of H. circularisquama and incubated until the host cell lysis was observed. The lysate was filtered through a 0.2 µm-pore-sized polycarbonate membrane filter (Whatman, Middlesex, U.K.) to remove host cell debris. The viral titer of the filtrate was estimated using the extinction dilution method as described previously (32).

**Virus inoculation**

An exponentially growing culture of H. circularisquama (25 ml) was inoculated with virus lysate at a multiplicity of infection (m.o.i.) of 10; and incubated. An aliquot of cell suspension (1.5 ml) was taken from the culture immediately after inoculation (0 h) and at 24, 48, and 72 h post inoculation (hpi). Host cells were centrifuged (13000 × g for 3 min), and the pellets were stored at −80°C for RNA extraction.

**Particle bombardment**

Viral RNA transfection of H. circularisquama cells was performed using the
Bio-Rad Biolistic PDS-1000/He Particle Delivery System (Bio-Rad Laboratories, Hercules, CA). The 0.6 µm-gold particles (Bio-Rad Laboratories) were coated with viral RNA as described below. HcRNAV virions were purified using the method as described previously (32). Viral RNA was isolated and purified using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The RNA concentration was determined spectrophotometrically, and its integrity was verified using 1.5 % denaturing agarose gel electrophoresis. Viral RNA (1.5 µg) was precipitated onto the gold particles (1.5 mg) by adding a 1/10 volume of 5 M ammonium acetate and 2 volumes of 2-propanol. The suspension was gently mixed and chilled at −20˚C for 1 h. After a quick centrifugation (2000 ×g for 3 s), the supernatant was discarded and the gold pellet was washed twice with 140 µl of 99.5% ethanol and re-suspended in 24 µl 99.5% ethanol. Six-µl was fixed on a macro carrier and dried.

Exponentially growing H. circularisquama cells (2.5 × 10^6 cells) were collected for particle bombardment on a quantitative filter paper No.3 (47 mm in diameter) (Advantec, Tokyo, Japan) using gravity filtration. The filter paper was set at approximately 6 cm below the microcarrier launch assembly and bombarded with the viral RNA-coated gold particles at a rupture pressure of 1350 psi in a vacuum of 28.5 inches Hg. After bombardment the filter paper was placed in fresh SWM3 medium (50 ml) and gently shaken to release transfected cells. The culture was incubated as described above. A 1.5 ml aliquot was sampled from the cell suspension immediately after transfection (0 h), and at 24 h and 48 h post transfection. Cells were pelleted by centrifugation (13000 ×g for 3 min) and stored at −80 °C prior to RNA extraction. The
remaining 40 ml culture was sampled 48 h post transfection and centrifuged at 860 × g for 10 min. The supernatant was filtered through a 0.2 µm-pore-sized polycarbonate membrane filter (Whatman) and used for the cell lysis assay (see below). The remaining cell pellet was fixed with 1% glutaraldehyde at 4˚C and processed for transmission electron microscopy (TEM). Preparation for TEM was performed as described previously (31, 32).

**Northern blot analysis**

Frozen cells were homogenized with plastic pestles in 100 µl RNase free water and 40 µl RNA extraction buffer (14). The aqueous phase was extracted using phenol prior to ethanol precipitation. Northern blot analysis of the purified RNA was performed as previously described (7). Strand-specific digoxigenin (DIG)-labeled RNA probes were transcribed from the plasmid pBSSK+MCP. The plasmid was constructed as follows: a cDNA fragment of HcRNAV34 from nucleotide 3182 to 4261 (containing the full sequence of ORF-2) was amplified using RT-PCR using the following primers:

34MCPBam1 5’-CGG GAT CCA TGA CCC GTC CCC TAG CTC TTA CC-3’ (the BamHI site is underlined) and 34MCPEco1 5’- CGG AAT TC T TAA GCA GCC ATC AAT GCT GGC ATA GC-3’ (the EcoRI site is underlined). The amplified DNA fragment was digested with BamHI and EcoRI and ligated into the corresponding restriction enzyme sites of pBluescript II SK+ (Stratagene, La Jolla, CA). DIG-labeled RNA probes specific for the (+) and (-)-strand of the HcRNAV genome were transcribed from the BamHI-linearized pBSSK+MCP with T7 RNA polymerase and
EcoRI-linearized pBSSK+MCP using T3 RNA polymerase according to the manufacturer’s protocols (Roche, Basel, Switzerland). The RNA signals were detected using a luminescence image analyzer (LAS-3000 mini; Fuji photo Film, Tokyo, Japan).

Cell lysis assay

The cell lysis assay was performed as described previously (32). Briefly, an aliquot (100 µl) of the 0.2 µm-filtered lysate from the viral RNA-transfected *H. circularisquama* culture was added to 8-wells of a 96 well round-bottom cell culture plate (Becton Dickinson Labware, Franklin Lakes, NJ) containing 150 µl of an exponentially growing host culture (HU9433-P or HCLG-1) and incubated. Cell lysis was monitored for 10 to 14 days using optical microscopy. Cell lysis was defined where >90 % of host cells were lysed.

RESULTS

Viral replication in host cells inoculated with HcRNAV

We used HCLG-1 and HU9433-P as representative host strains for HcRNAV109 (type CY) and HcRNAV34 (type UA), respectively (30). To determine whether viral genomic RNA can replicate in incompatible host/virus combinations, the accumulation of viral RNA was examined using Northern blot analysis with a (+)-strand specific RNA probe (Fig. 2A). In HCLG-1 and HU9433-P cultures respectively inoculated with HcRNAV109 and HcRNAV34, respectively (the compatible combinations), accumulation of viral genomic RNA (4.4kb) was detected at 24, 48 and 72 hpi (Fig. 2A).
In contrast, in the HU9433-P culture inoculated with HcRNA V109 and HCLG-1 culture inoculated with HcRNA V34 (the incompatible combinations), no (+)-strand RNA accumulation was detected (Fig. 2A). When the membrane was exposed for longer, a faint band of viral genomic RNA (4.4 kb) was detected in the lanes of incompatible host/virus combinations. However, faint bands were also visible immediately after inoculation with either compatible or incompatible host/virus combinations (Fig. 2B). To determine if these faint signals were due to the initial virus inoculum, we analyzed the accumulation of the complementary (-)-strand RNA that is specifically synthesized during the replication process of the positive-strand RNA virus (2) using the (-)-strand-specific RNA probe (Fig. 2C). Genomic length (-)-strand RNAs (4.4 kb) were observed only in the compatible combinations, but not in incompatible combinations using intensive exposure. This suggests the viral RNA was not replicated in incompatible host/virus combinations where viruses were inoculated by simply adding a virus suspension to the host culture.

Four additional RNA bands (10 kb, 2.6 kb, 1.8 kb and 1.2 kb) other than HcRNA V genomic RNA (4.4 kb) were observed in Northern blot analysis (Fig. 2A and C).

**Viral RNA replication in host cells transfected with HcRNA genomic RNA**

To determine whether incompatible *H. circularisquama* cell is permissive for HcRNAV replication, a direct transfection of viral RNA into *H. circularisquama* cells was performed using particle bombardment (Fig. 3). For a negative control, we added viral RNA-coated gold particles and detected no RNA signal corresponding to the
complementary strand of HcRNAV genomic RNA in the *H. circularisquama* cells (data not shown). This indicates the purified viral RNA was not contaminated with infectious virions. In HU9433-P and HCLG-1 transfected respectively with purified genomic RNA of HcRNA34 and HcRNA109 (the compatible combinations), RNA corresponding to the complementary strand of genomic RNA (4.4 kb) was observed (Fig. 3). We also observed complementary strand of genomic RNA (4.4 kb) in incompatible host/virus combinations (i.e. HU9433-P transfected with HcRNA109 RNA; and HCLG-1 transfected with HcRNA34 RNA) where host cells were transfected with viral RNA using particle bombardment (Fig. 3). This shows *H. circularisquama* cells are permissive for HcRNAV replication in incompatible host/virus combinations as well as with compatible combinations. Hence, it is therefore unlikely the intraspecies host specificity of HcRNAV is determined during the viral RNA replication process. While, it should be noted that the RNA accumulation levels were different among host/virus combinations. Further quantitative analysis will be required to investigate whether the efficiency of viral replication differs among host/virus combinations.

**Properties of virions produced in incompatible host cells**

To determine whether progeny virions were formed in *H. circularisquama* cells that were directly transfected with purified HcRNAV RNA, intracellular accumulation of HcRNAV-like particles was examined for using TEM. Although no HcRNAV-like particle was observed in healthy cells (data not shown), crystalline arrays or unordered aggregations of HcRNAV-like particles were observed both with compatible (i.e.
HU9433-P transfected with HcRNA V34 RNA and HCLG-1 transfected with 
HcRNA109 RNA) and incompatible (i.e. HU9433-P transfected with HcRNA109 
RNA and HCLG-1 transfected with HcRNA V34 RNA) host/virus combinations (Fig. 4). 
The 0.2 µm filtrate of cell cultures transfected with HcRNA V34 genomic RNA 
caused lysis of HU9433-P cells but not HCLG-1 cells (Table 1). Similarly, the 0.2 µm 
filtrate of cell cultures transfected with HcRNA109 genomic RNA caused lysis of 
HCLG-1 cells but not HU9433-P cells (Table 1). This suggests the progeny viruses 
produced in transfected *H. circularisquama* cells retained the original infection 
specificity encoded by the imported viral RNA.

**DISCUSSION**

**Infection steps determining the intraspecies host specificity**

When HcRNA virions were simply added to *H. circularisquama* cultures, viral 
RNA replication, propagation of progeny virions and host cell lysis occurred with 
compatible host/virus combinations but not with the incompatible combinations (Fig. 2) 
(32). When viral RNA was transfected into *H. circularisquama* cells by particle 
bombardment, viral RNA replication occurred with both compatible and incompatible 
host/virus combinations (Fig. 3). In addition, infectious progeny virus was produced 
both in incompatible host/virus combinations (Fig. 4 and Table 1). This indicates 
intraspecies host specificity of HcRNAV is not determined by the viral RNA replication 
and the encapsidation process. Instead, it is determined by the upstream events; most 
probably the specific binding of virus to the host cell surface receptor, entry into host
cell and disassembly of viral particles.

The upstream events of non-enveloped RNA viruses are not sufficiently understood except for poliovirus and its relatives (8, 27). Poliovirus infection is initiated when virus particles attach to specific cell-surface receptors (17). Virus-receptor interaction induces irreversible conformational change in the virus capsid (33, 34) and producing a particle with an altered sedimentation property, antigenicity, and sensitivity to proteases. This meta-stable particle further undergoes a secondary conformational change in which the viral RNA is ejected into the cytoplasm (9). However, the factors triggering RNA release are unknown. This receptor-induced conformational change in the viral particle is also proposed for Flock house virus (genus Nodaviridae)(35). Our results suggest specific binding of a virus particle to its corresponding host cell surface receptor may be the primary determinator for intraspecies host specificity of HcRNAV.

Possible molecular mechanism for intraspecies host specificity of HcRNAV

Two remarkable differences were observed between HcRNAV34 and HcRNAV109 genomes at the nucleotide sequence level (20). One is the 15 nucleotide deletion in the HcRNAV34 ORF-1 (Fig. 1). However, no relationship between this deletion and intraspecies host specificity is predicted (Y. Tomaru. unpublished data). The other difference is the highly frequent nucleotide substitutions in ORF-2 (variable region I – IV) that cause amino acid substitutions in the CP (Fig. 1). A tertiary structure prediction of the CP shows most of the substituted amino acid residues to be located on exterior surface of the virion (20). Therefore, the amino acid substitutions in the CP
likely alter the host specificity of HcRNAV.

Similar phenomena are also observed in other virus studies. For example, canine parvovirus is a host range variant of feline parvovirus that acquired the ability to infect dogs through changes in surface amino acid residues in its capsid protein VP2 (3, 22). Furthermore, a single amino acid substitution in the VP2 altered the host cell specificity (21) and the specific binding ability for the transferrin receptor (10). With influenza virus, human viruses preferentially bind to cell surface oligosaccharides that contain the 5-N-acetylneuraminic acid-\(\alpha\)-2,6-galactose (Neu5Aca\(\alpha\)2,6Gal) linkage, while avian and equine viruses bind to Neu5Aca\(\alpha\)2,3Gal (24). Ito et al. (12) found if human influenza virus was passed in allantoic cavities of embryonated chicken eggs containing Neu5Aca\(\alpha\)2,3Gal but not Neu5Aca\(\alpha\)2,6Gal, progeny virions acquired a Neu5Aca\(\alpha\)2,3Gal specificity. This change in receptor specificity was accompanied with amino acid substitutions in the receptor-binding site of the hemagglutinin molecule (12).

What is the virus receptor of *H. circularisquama*? In many cases, the external portions of membrane proteins have a complex of branched carbohydrate chains and such membrane glycoproteins are frequently used as viral receptors (26). Costas and Rodas (5) reported the binding spectra of fluorescence-labelled lectins to marine dinoflagellates varied not only at the species level (i.e. between the toxic *Gymnodinium catenatum* and the nontoxic *Gymnodinium* sp.) but also at the clone level (e.g. *Alexandrium minutum* and *A. excavatum*). We speculate cell surface glycoprotein composition of *H. circularisquama* may vary between ecotypes and this difference is implicated in intraspecies host specificity of HcRNAV.
Characteristics of HcRNA V replication

In this study, we analyzed the accumulation of negative-strand viral RNA as a marker of viral replication. In addition to the genomic length RNA band (4.4kb), an intense RNA band (1.2 kb), and three weak RNA bands (10 kb, 2.6kb and 1.8 kb) were also observed (Fig. 2C and 3), and corresponding sized RNA species positively reacted with (+)-strand specific probe (Fig. 2A). Based on the nucleotide sequence, expected size for ORF2 subgenomic RNA is >1.2 kb. In addition, we have a preliminary data that the (+)-strand 1.2kb band was detected by RNA probe contained ORF2 sequence but not by probe contained ORF1 sequence (H. Mizumoto and K. Nagasaki, unpublished data). It is possible that the 1.2kb-long RNA corresponds to subgenomic RNA for ORF-2.

Transcription of the viral subgenomic RNAs is a strategy frequently used by eukaryotic positive-strand RNA viruses. Subgenomic RNA mediates the expression of the 3’-proximal ORF on multicistronic genomic RNA because only the first ORF on the eukaryotic mRNA is translated. Three general mechanisms of subgenomic RNA synthesis are proposed (18). In one of these mechanisms, premature termination occurs during negative-strand synthesis from the genomic RNA template. This results in synthesis of a subgenomic length negative-strand RNA (36), as observed with HcRNAV (Fig. 2C). In contrast to the accumulation of negative-strand RNAs (Fig. 2C), intense positive-strand RNA signals except for genomic RNA were not observed (Fig. 2A). It may be that subgenomic RNAs corresponding to the accumulated negative-strand
RNAs were synthesized only at earlier stages during replication as observed with *Flock house virus* (37). Further analysis will shed light on the replication and gene expression mechanisms of HcRNAV.

When viral RNA was transfected into *H. circularisquama* cells by particle bombardment, propagation of progeny virions occurred both in compatible and incompatible host/virus combinations (Fig. 3, 4, and Table 1). This indicates all cellular factors essential for HcRNAV replication, transcription, translation and encapsidation are present in both *H. circularisquama* isolates. However, it should be noted that the RNA accumulation levels differed among host/virus combinations (Fig. 3). The (+)-strand specific RNA probe used here was synthesized using HcRNAV34 genomic RNA as a template where it hybridized with similar intensity to both of the genomic RNAs of HcRNAV34 and HcRNAV109 (Fig. 2). This suggests the cells permitting virus production may have different levels of permissiveness; i.e., some *H. circularisquama* isolates may produce large amounts of virus while others may permit only a low level of viral replication. Additionally, with *H. triquetra* cells (another species belonging to the genus *Heterocapsa*) transfected with the HcRNAV RNA, accumulation of viral negative-stranded RNAs was not detected (H. Mizumoto, Y. Tomaru and K. Nagasaki, unpublished data). This suggests the intracellular condition of *H. triquetra* is not suitable for replication of HcRNAV. Therefore, the mechanisms determining host specificity of dinoflagellate RNA viruses are considered to be different at the intraspecies and interspecies level.
ACKNOWLEDGEMENTS

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REFERENCES


Table 1. Lytic activity of 0.2 µm-filtrate of viral RNA-transfected *Heterocapsa circularisquama* cells.

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<th>Combination of viral RNA and transfected host clone</th>
<th>Lytic activity&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td>HcRNA V34 RNA → HU9433-P</td>
<td>+</td>
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<tr>
<td>HcRNA V34 RNA → HCLG-1</td>
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<tr>
<td>HcRNA V109 RNA → HCLG-1</td>
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<sup>a</sup> +, Cell lysis occurred in all 8-wells of a culture plate; -, no effect.
Figure Legends

Fig. 1. Schematic diagram of HcRNA V34 and HcRNA V109 genome structure (20).

The HcRNA V genome is shown as a thick line with the protein coding regions depicted as boxes. Filled box indicate a nucleotide (nt) deletion region. Shaded boxes indicate high frequency nucleotide substitution regions.

Fig. 2. Accumulation of positive- (A and B) and negative-strand RNA (C) of HcRNA V in Heterocapsa circularisquama cells inoculated with HcRNA V34 and HcRNA V109 virion. Total RNA was extracted from H. circularisquama cells immediately after inoculation (0 h) and 24, 48 and 72 h post inoculation (hpi), separated by gel electrophoresis, and blotted onto membranes. The membranes were then probed with strand-specific DIG-labeled RNA probes. An equal amount of genomic RNA of HcRNA V34 or HcRNA V109 was used as a positive control. Length of RNAs is indicated on the right. Total RNA stained with SYBR Gold is shown in (D).

Fig. 3. Accumulation of negative-strand HcRNA V RNA in Heterocapsa circularisquama cells transfected with HcRNA V34 RNA or HcRNA V109 RNA. Total RNA was extracted from H. circularisquama cells immediately after inoculation (0 h) and 24 h and 48 h post inoculation (hpi), separated by gel electrophoresis, and blotted onto membranes. The membranes were then probed with negative-strand specific DIG-labeled RNA probe. Length of RNAs is indicated on the right. Total RNA stained
with SYBR Gold is shown in (D).

Fig. 4. Transmission electron micrographs of *Heterocapsa circularisquama* cells 48 h post transfection with HcRNAV34 RNA or HcRNAV109 RNA. Note the propagation of HcRNAV-like particles forming crystalline array and/or unordered aggregation in the cytoplasm. Bars indicate 200 nm.
Fig. 1.

Variable regions

HcRNAV34 (Type UA)

19
15 nt deletion (1152-1166)

ORF1

HcRNAV109 (Type CY)

19

ORF2

Variable regions

I II III IV
**Fig. 3.**

### HCRNAV 109

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(Host isolate) HU9433-P HCLG-1

- strand

Total RNA

### HCRNAV 34

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(Host isolate) HU9433-P HCLG-1

- strand

Total RNA
Transfected viral RNA

HcRNAV109

HcRNAV34

HCLG-1

HU9433-P

Host isolate

Fig. 4.