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Cryo-electron microscopy of nodavirus RNA replication organelles illuminates positive-strand RNA virus genome replication

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The nodavirus flock house virus recently provided a well-characterized model for the first cryo-electron microscope tomography of membrane-bound, positive-strand RNA ((+) RNA) virus genome replication complexes (RCs). The resulting first views of RC organization and complementary biochemical results showed that the viral RNA replication vesicle is tightly packed with the dsRNA genomic RNA replication intermediate, and that (+)ssRNA replication products are released through the vesicle neck to the cytosol through a 12-fold symmetric ring or crown of multi-functional viral RNA replication proteins, which likely also contribute to viral RNA synthesis. Subsequent studies identified similar crown-like RNA replication protein complexes in alphavirus and coronavirus RCs, indicating related mechanisms across highly divergent (+)RNA viruses. As outlined in this review, these results have significant implications for viral function, evolution and control.

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Introduction

The COVID-19 pandemic and many other established and emerging viral diseases – including polio, hepatitis A and C, Zika and dengue fevers, chikungunya, and so on – are caused by positive-strand RNA ((+)RNA) viruses, whose virions carry a messenger-sense RNA genome and replicate by RNA intermediates without reverse transcription. (+)RNA viruses are the largest genetic

category of eukaryotic viruses and include numerous plant, animal and human pathogens. The massive public health and socio-economic impacts of SARS-CoV-2 are a stark reminder of the desperate need for more powerful means to control such viruses. Rational development of such improved controls requires improved understanding of (+)RNA virus replication and host interactions [1,2].

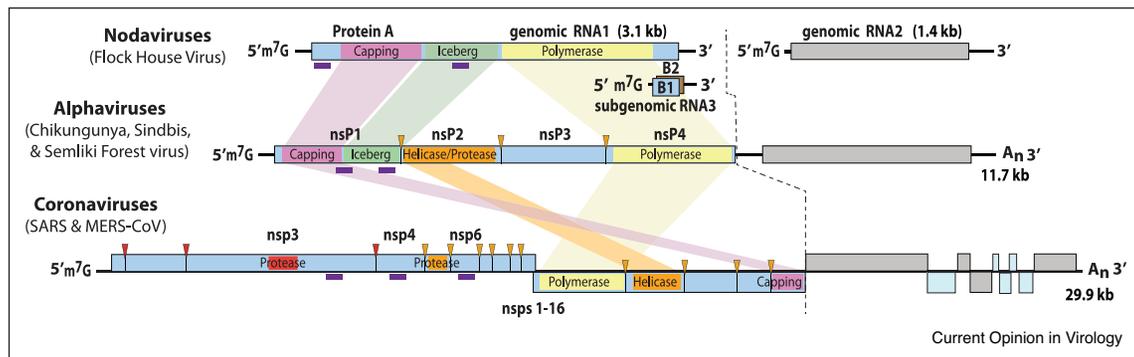
(+)RNA viruses devote most of their protein coding capacity (often 70% or more) to genome replication, a process at the heart of the viral life cycle (Figure 1). Despite substantial variation in other features, all (+)RNA viruses remodel host membranes to build RNA replication complexes (RCs) [3,4]. These RCs use virus-induced, 50–300 nm diameter vesicles to concentrate viral proteins and RNA, compartmentalize viral RNA synthesis from competing processes such as translation, and protect double-stranded RNA replication intermediates against host innate immune defenses.

Two major classes of (+)RNA virus RCs are known [5]. Spherular membrane invaginations, called spherules, are used by alphaviruses, nodaviruses, flaviviruses, and many other (+)RNA viruses [3,4]. Double-membrane vesicles (DMVs) are formed by beta-coronaviruses such as SARS-CoV-2, by the flavivirus-related hepatitis C virus, and during late stages of the picornavirus life cycle [6–10].

Nodaviruses as model (+)RNA viruses

The nodavirus Flock House Virus (FHV) is a relatively simple, high-yielding, well-characterized (+)RNA virus [11]. FHV infects *Drosophila* cell lines and FHV RNA, when transfected into cells, launches productive replication in an amazingly broad spectrum of hosts including yeast, *Caenorhabditis elegans*, mammalian and plant cells. FHV’s two component, ~4.5 kb genome expresses four known proteins (Figure 1). RNA1 encodes protein A, a 998 aa, multi-functional protein with membrane-association, multimerization, RNA-dependent RNA polymerase and 5’ RNA capping activities [12–14]. RNA1-derived subgenomic RNA3 encodes proteins B1 and B2. B1, a small nuclear protein of incompletely understood function colinear with protein A’s C-terminus, is dispensable for replication in cell culture [15]. B2 is a dsRNA-binding protein that inhibits host RNA interference [16,17]. RNA2 encodes capsid protein. FHV’s simple structure, embodiment of widely conserved features (see below), high yield and

Figure 1



Positive strand RNA virus genome maps.

Schematic representation of the nodavirus, alphavirus, and coronavirus genomes. Their 4.4 kb, 11.7 kb and 30 kb genomes, respectively, are shown progressively scaled down for ease of presentation and alignment. A vertical dashed line presents the general separation of RNA replication protein (left) and other protein (right) coding regions, to emphasize that the essential RNA replication functions to the left of the line expend up to 70% or more of genome coding capacity. Colored highlights indicate regions of RNA replication protein functional and structural similarity. Proteolytic cleavage sites are indicated with triangles. Membrane-association domains are underlined in purple. Virion protein coding regions are indicated in gray.

tractability have made it a well-studied model for RNA replication, encapsidation, and other processes [11].

Cryo-EM tomography of FHV RCs

FHV remodels outer mitochondrial membranes (OMMs) to form spherular RC vesicles that fill and dilate the space between the OMM and inner mitochondrial membrane [18]. In the first three dimensional (3D) electron microscope (EM) tomography of a (+)RNA virus RC, nodavirus RCs were revealed as clustered, ~50 nm diameter vesicular invaginations of the OMM, each connected to the cytosol via an ~10 nm diameter necked pore (Figure 2a) [19]. While these classical 3D EM studies resolved the membrane topology of nodavirus RCs, visualizing the RNA and protein components required cryo-electron microscopy (cryo-EM), free from the limitations and artefacts of classical EM chemical fixation and heavy metal staining [20–22]. An early two-dimensional cryo-EM study of FHV RCs began to characterize heterogeneity in RC sizes and, building on prior indications of filamentous density inside chemically fixed RC spherules [18,23], showed swirls of density within RC vesicles [24]. Soon after, more powerful 3D cryo-EM tomography provided a comprehensive, higher-resolution 3D view of the FHV RC, revealing that the RC spherule vesicle is densely packed with continuously coiled fibrils (Figure 2b) [25].

Multiple results show that these interior filaments represent viral dsRNA replication intermediates. First, FHV dsRNA is exclusively membrane-associated and protected from nucleases until treatment with membrane-disrupting detergents [19,24]. Moreover, plasmid expression of protein A and defined RNA templates produces active RCs whose vesicle sizes are strongly correlated

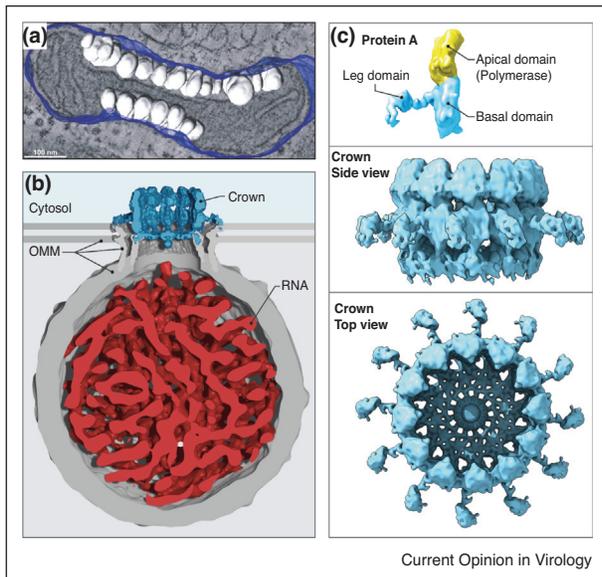
with RNA template length over the complete, nearly 10-fold range tested (3.1–0.4 kb) [25]. RNA packaging density calculations imply that most spherules contain a single dsRNA replication intermediate [25]. Similar dependence of RC vesicle size on RNA template length has been found for Semliki Forest alphavirus [26].

Crown complexes of viral RNA replication proteins

By visualizing unstained, native electron density in higher resolution 3D structures, cryo-EM tomography of mitochondria from FHV-infected cells revealed many other previously unrecognized features of (+)RNA virus RCs. Perhaps most striking was the finding that the cytosolic side of each RC's necked membrane channel to the cytoplasm was 'crowned' with a ringed complex (Figure 2b) that contained high levels of multifunctional FHV RNA replication protein A [25]. Subtomogram averaging showed that each crown contains a 12-fold symmetric, cupped, inner turret of ~19 nm diameter, with 12 flanking projections at ~35 nm diameter [25]. Positioning of this membrane-bound crown at the spherule neck answered the long-standing question of what stabilizes the high energy RC structure. The high order 12-fold symmetry of the crown may in part be an adaptation to provide sufficient strength to clamp the vesicle neck against the pressure of the tightly-packed, electrostatically-repulsing dsRNA within the curved membrane spherule.

The major products of (+)RNA virus RCs are positive-strand genomic ssRNAs, which are synthesized in ~100-fold excess to (–)RNAs and delivered to the cytosol for translation and encapsidation [19]. Consistent with this, cryo-EM tomography showed that FHV RC crowns are

Figure 2



Nodavirus RNA replication complexes.

(a) 3D tomographic reconstruction of a mitochondrion in a chemically fixed, FHV-infected *Drosophila* S2 cell (from Ref. [19]). The outer mitochondrial membrane (OMM) is outlined in blue, and FHV RC spherules in white. (b) Cryo-EM reconstruction of an FHV RC spherule on the OMM crowned at the aperture by a multimeric ring of protein A (modelled after Ref. [25]) with the higher resolution crown and membrane neck structure from Ref. [27] included. (c) Detailed views at 8.5 Å resolution of (top panel) an individual subunit revealing distinct apical and basal globular domains and a leg-like extension, and (lower panels) side and top views of the protein A 12-fold symmetric crown (based on Ref. [27]).

frequently the origins for cytosolic filaments presumably representing progeny (+)RNA genomes [25]. In addition to gating the release of nascent product RNA, crowns likely have important roles in viral RNA synthesis, since they contain the viral protein A RNA-dependent RNA polymerase and RNA capping domains and are strategically positioned at the interface between the archival viral dsRNA template and nascent progeny (+)RNAs.

A further foundation for mechanistic insights into such crown functions was provided by recent striking improvement of crown structure resolution from ~33 Å [25] to ~8.5 Å [27]. This advance, achieved by improved sample preparation, imaging and subtomogram averaging, showed the crown to consist of 12 vertical subunits connected into a ring by multiple lateral interactions (Figure 2c). In turn, each vertical subunit consists of an apical lobe supported by a membrane-interacting basal lobe from which a smaller 'leg' extends radially to also bind the membrane [27]. The resulting two rings of OMM contacts strongly constrain the otherwise fluid membrane, causing the spherule's membranous neck to appear as a tightly focused lipid bilayer in subtomogram averages of thousands of RCs (Figure 2b and Ref. [27]).

Multiple lines of evidence indicate that viral protein A (Figure 1) is the major, if not sole component of the FHV crown. Protein A is the only FHV protein required for RNA replication [23]. A C-terminal protein A epitope tag adjacent to the polymerase domain mapped to the top of the apical lobe, showing that crowns are rings of 12 copies of protein A, and the ~150 nm³ volume of each crown subunit is quite sufficient to contain the predicted volume of protein A [27]. The side-to-side interactions joining apical to apical and basal to basal lobes to form the crown ring are consistent with previously mapped multiple protein A multimerization domains [12].

The above epitope tag mapping shows that the protein A polymerase domain occupies the apical lobe, matching the predicted polymerase structure [27]. Protein A's membrane interaction sites [27,28] and further considerations outlined below suggest that the basal region may represent the RNA capping and 'iceberg' domains and the leg may include the N-terminal transmembrane region (Figure 1).

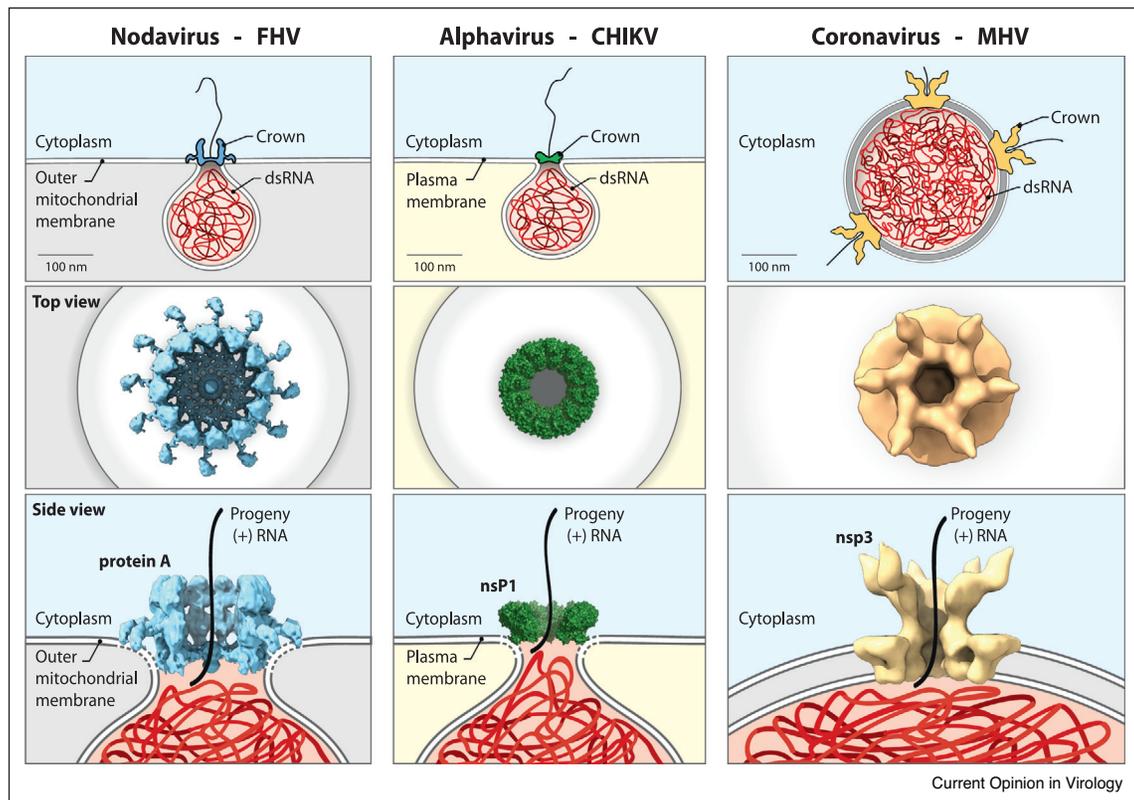
Parallels with alphaviruses and coronaviruses

Comparisons of predicted secondary structures identified unsuspected similarities between N-proximal RNA capping domains and downstream membrane-interacting domains in the RNA replication proteins of nodaviruses like FHV and members of the large alphavirus-like superfamily (Figure 1 and Ref. [28]). Coupled with related polymerase regions, this shows that the nodavirus and alphavirus RNA replication machineries are related and differentiated primarily by an NTPase/helicase domain present in alphaviruses but missing in nodaviruses. Consistent with this, viruses in the alphavirus superfamily make invaginated spherule RCs similar to those of FHV.

As shown in Figure 1, alphavirus non-structural protein nsP1 thus corresponds to the RNA capping and 'iceberg' membrane interaction regions of FHV protein A, which as noted above may represent the basal region of the FHV crown (Figure 2c). In confirmation of this similarity, exogenously expressed chikungunya alphavirus nsP1 assembles into a 12-mer ring with a central pore (Figure 3 and Refs. [26,27]). This nsP1 ring is proposed to sit on the spherule neck in a fashion remarkably similar to the basal ring of the FHV crown (Figure 3).

Coronaviruses are evolutionarily quite distant from alphaviruses and nodaviruses (Figure 1) and as mentioned earlier form DMV rather than spherule RCs. Nevertheless, cryo-EM tomography shows that coronavirus DMVs also bear a crown-like membrane-spanning channel (Figure 3, right column) connecting the DMV interior to the cytosol and presumably serving as the long-sought

Figure 3



Comparison of nodavirus RCs and crowns to those of alphaviruses and coronaviruses.

Left column: the spherule RNA replication complexes of nodaviruses are crowned by 12-fold symmetric crown complex of protein A [27**].

Middle column: In the absence of other viral proteins, exogenously expressed alphavirus nsP1, which contains RNA capping domains similar to those of the nodavirus crown basal region, assembles into a 12-mer ring that is proposed to reside atop alphavirus spherules similarly to the basal ring of the nodavirus crown [29**,30**]. In active alphavirus RNA replication complexes, this nsP1 ring presumably represents the base of a larger complex that also contains additional alphavirus RNA replication proteins nsPs 2–4 (Figure 1).

Right column: Coronaviruses replicate their RNA in double-membrane vesicles (DMVs) rather than invaginated spherules, but nevertheless also have crown-like protein apertures connecting the DMV interior to the cytosol [31**]. These coronavirus crowns contain viral protein nsp3 and additional undefined viral or cellular proteins. Emerging results suggest that coronavirus crowns also engage in dynamic interactions with other viral proteins and RNAs to mediate RNA synthesis and encapsidation.

channel for product RNA release [31**]. The coronavirus crown contains viral RNA replication protein nsp3 and other undetermined viral or host proteins, and may also serve as a scaffold for transient interactions of other viral RNA replication and virion proteins [31**]. The coronavirus crown also has 6-fold rather than 12-fold symmetry, which may reflect some variation in function, evolution or both. Nevertheless, the presence in coronavirus DMVs of a ringed multimeric channel for product RNA release, and likely also involved in RNA synthesis, shows that crucial principles of RNA replication and RC function are conserved across spherule- and DMV-forming (+)RNA viruses [5].

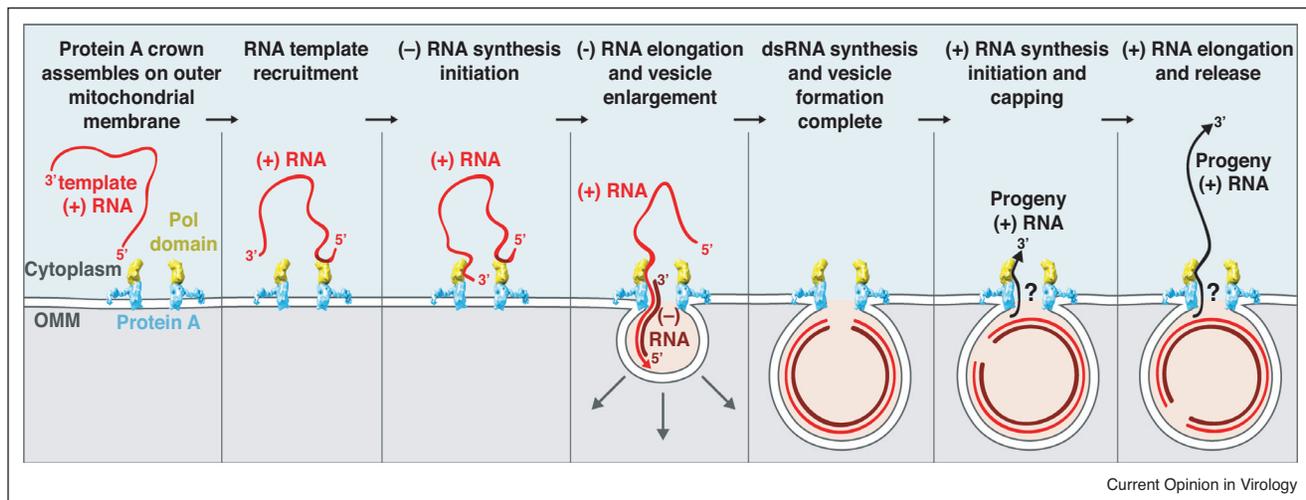
Mechanistic models and future directions

FHV crown structures to date have been derived from mature RCs that have already undergone (–)RNA synthesis to form dsRNA templates, but mutational and

other studies show that distinct earlier states exist associated with RC assembly and maturation. Based on these results, Figure 4 presents an FHV RC assembly and function model [25**,27**] in which protein A associates with the OMM [13], assembles into a 12-fold symmetric crown [12,27**], recruits a founding viral (+)RNA template by interactions of specific protein A and viral RNA elements [32,33], directs (–)RNA synthesis and concomitant formation of the spherule replication vesicle [23], and so on.

Like all models, this emerging view provides a conceptual platform to generate and test new questions. One issue is that the apical polymerase domain (Figure 2c) appears well positioned for its role in (+)RNA template recruitment [32] and (–)RNA synthesis (Figure 4), but difficult to reconcile with (+)RNA synthesis due to its separation from the vesicle-protected dsRNA template. Possible

Figure 4



Model for nodavirus genome replication complex formation and function.

Nodavirus Protein A associates with the outer mitochondrial membrane (OMM), assembles into a 12-fold symmetric crown, and recruits a founding genomic (+)RNA template. Negative-strand RNA synthesis induces the formation of spherular invaginations, possibly by filling the growing vesicle with the dsRNA product of (-)RNA synthesis. Upon completion of (-)RNA synthesis, spherules reach full final size, and the replication complex switches to repetitive synthesis and capping of (+)RNA genome copies that are released into the cytosol. The question marks in the two rightmost panels indicate that certain issues regarding (+)RNA synthesis and release are under further study, including whether RNA polymerase activity for (+)RNA synthesis is provided by protein A in the crown or in another form.

resolutions include a dramatic protein A conformational change not yet visualized or a second non-crown pool of protein A inside or below the crown's central turret. Asymmetric densities are often seen in this region [27**] and a polymerase there would be better positioned to interact with the dsRNA template and direct product (+)RNA to the basal capping domain while exiting to the cytosol. Such a basal polymerase would further extend parallels with transcription complexes at the fivefold axes of dsRNA reovirus cores, in which a basal polymerase copies a packaged dsRNA template into (+)RNA products that exit to the cytosol through a ringed turret of RNA capping proteins [34,35]).

Among numerous other questions, for example, do additional viral or host proteins contribute to currently unassigned RC densities such as the central floor-like density at the OMM level of the crown (Figure 2b–c)? As suggested by recent coronavirus results [31**], do nodavirus and alphavirus crowns serve as docking sites for virion proteins to promote encapsidation of newly synthesized genomic RNAs? Can the expanding grasp of structure, function and conserved principles in (+)RNA virus RCs be leveraged to provide improved, generalizable or even broad-spectrum antiviral strategies against relevant viral and/or host proteins such as those involved in RC membrane synthesis and remodeling? Accelerating progress on these and other questions should continue to advance understanding and control of the critically important (+) RNA viruses.

Conflict of interest statement

Nothing declared.

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