ANNUAL REVIEWS

Annual Review of Virology

Crowning Touches in Positive-Strand RNA Virus Genome Replication Complex Structure and Function

Masaki Nishikiori,[1,2] Johan A. den Boon,[1,2] Nuruddin Unchwaniwala,[1,2,3] and Paul Ahlquist[1,2]

[1]John and Jeanne Rowe Center for Research in Virology, Morgridge Institute for Research, Madison, Wisconsin, USA; email: ahlquist@wisc.edu

[2]Institute for Molecular Virology and McArdle Laboratory for Cancer Research, University of Wisconsin–Madison, Madison, Wisconsin, USA

[3]Current affiliation: Assembly Biosciences, Inc., South San Francisco, California, USA

Annu. Rev. Virol. 2022. 9:193–212

First published as a Review in Advance on May 24, 2022

The *Annual Review of Virology* is online at virology.annualreviews.org

https://doi.org/10.1146/annurev-virology-092920-021307

Keywords

positive-strand RNA virus, RNA replication complex, cryo-EM tomography, crown complex, nodavirus, alphavirus, coronavirus

Abstract

Positive-strand RNA viruses, the largest genetic class of eukaryotic viruses, include coronaviruses and many other established and emerging pathogens. A major target for understanding and controlling these viruses is their genome replication, which occurs in virus-induced membrane vesicles that organize replication steps and protect double-stranded RNA intermediates from innate immune recognition. The structure of these complexes has been greatly illuminated by recent cryo-electron microscope tomography studies with several viruses. One key finding in diverse systems is the organization of crucial viral RNA replication factors in multimeric rings or crowns that among other functions serve as exit channels gating release of progeny genomes to the cytosol for translation and encapsidation. Emerging results suggest that these crowns serve additional important purposes in replication complex assembly, function, and interaction with downstream processes such as encapsidation. The findings provide insights into viral function and evolution and new bases for understanding, controlling, and engineering positive-strand RNA viruses.

(+)RNA: positive-strand RNA

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2

HCV: hepatitis C virus

dsRNA: double-stranded RNA

RC: replication complex

DMV: double-membrane vesicle

1. INTRODUCTION

Positive-strand RNA viruses [(+)RNA viruses] are one of the most numerous and devastating viral classes. Their members include severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), cause of the ongoing coronavirus disease 2019 (COVID-19) pandemic, the tumor-inducing hepatitis C virus (HCV), paralytic poliovirus, lethal encephalitis and hemorrhagic fever viruses, and many other human, animal, and plant pathogens (1, 2). In parallel, these viruses have demonstrated considerable potential for beneficial uses in medicine, biotechnology, and nanotechnology through their abilities to deliver, dramatically amplify, express, and evolve selected genes and RNA elements (e.g., 3). The practical potential of such RNA pathways has become even more apparent with the recent successes of messenger RNA (mRNA) vaccines (4) and increasingly numerous and powerful RNA-based tools for gene editing and regulation (5–7).

Better understanding of replication and pathogenesis is crucial to (+)RNA virus control, to predicting the emergence of future dangerous viral species and variants, and to improving engineered uses of viral derivatives and pathways. One essential target for understanding, controlling, and employing (+)RNA viruses is their genome replication. Such RNA replication invariably uses most of the viral genome coding capacity—e.g., greater than 70% of the SARS-CoV-2 genome—and thus is arguably the main job of the virus. This (+)RNA virus genome replication proceeds solely through RNA intermediates, without any natural DNA forms (**Figure 1*a***). In eukaryotes, these processes occur in virus-induced, membrane-bounded mini-organelles that organize RNA replication steps and protect double-stranded RNA (dsRNA) replication intermediates from innate immune recognition. Two classes of such RNA replication complexes (RCs) are known. The first, designated spherules, are ~50- to 150-nm invaginations of an intracellular membrane, whose interior remains connected to the cytosol by a necked channel (**Figure 1*b***). The second class consists of ~200- to 300-nm double-membrane vesicles or DMVs, which lack any membranous opening to the cytosol (**Figure 1*c***).

Prior ultrastructural knowledge of (+)RNA virus RCs had been limited to their membrane architecture. Recent application of cryo-electron microscopy (cryo-EM) and complementary approaches to a few pioneer systems has greatly advanced understanding of these complexes and their functions by revealing the location, organization, and interactions of viral RNA templates, RNA replication proteins, and other relevant viral and cellular factors. In this review we summarize these and other recent breakthroughs in (+)RNA virus genome replication and their implications for viral function and control. Major findings include that, in addition to enzymatic roles in viral RNA synthesis, (+)RNA virus replication proteins have key structural roles in RCs. For both spherule and DMV RCs, these viral proteins form ringed complexes or crowns that among other functions serve as cytosolic exit channels for progeny (+)RNA products. As outlined below, such crowns appear to represent a crucial nexus for many infection steps, from initial recruitment of RNA replication factors and templates through successive RNA replication functions, post-translational maturation steps, encapsidation, and host innate immune interactions. Ongoing analysis of these crowns promises to provide further valuable insights regarding RC structure, operation, assembly, and evolution.

2. SPHERULE RNA REPLICATION COMPLEXES

Below we introduce some general RC principles and emerging specifics of RCs employing spherule invaginations as RNA replication compartments (**Figure 1*b***), using several of the best-characterized examples among the many (+)RNA viruses that employ this style of RC.

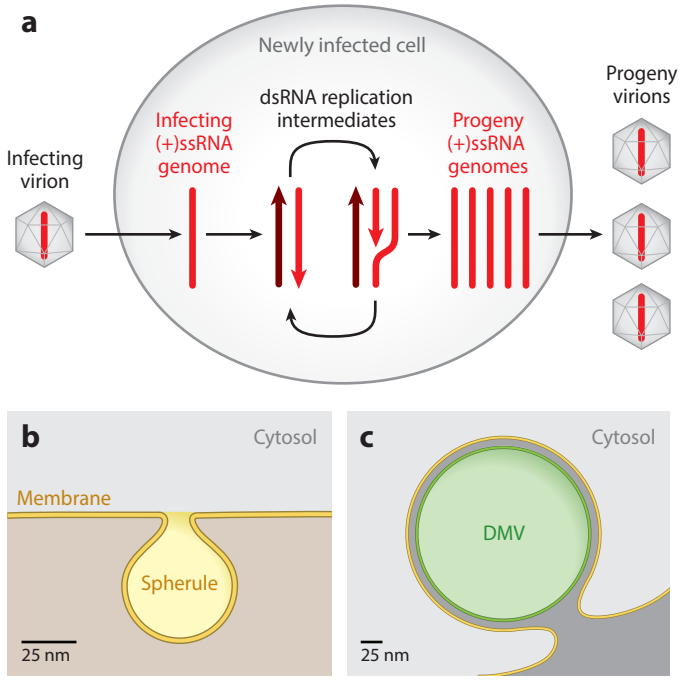

Figure 1

(+)RNA virus genome replication and associated membrane compartments. (*a*) Schematic of (+)RNA virus genome replication showing the progression through key replication intermediates. An infecting virion releases a messenger-sense RNA genome into the cytoplasm. Viral nonstructural RNA replication proteins synthesize a complementary (−)RNA. Resulting dsRNA replication intermediates function as templates for progeny (+)RNA genomes. The asymmetry of RNA synthesis typically produces a (+)RNA:(−)RNA ratio of approximately 50–100. Depending on the virus, progeny virions may be released from infected cells in lytic and/or nonlytic fashions. (*b*) Schematic representation of (+)RNA virus spherule RNA replication compartments. Such spherules are invaginations of various intracellular organellar membranes and are typically 50–100 nm in diameter. (*c*) Schematic representation of (+)RNA virus DMV RNA replication compartments, which are typically 200–300 nm in diameter. Abbreviations: DMV, double-membrane vesicle; dsRNA, double-stranded RNA; (−)RNA, negative-strand RNA; (+)RNA, positive-strand RNA; ssRNA, single-stranded RNA.

2.1. Nodaviruses

Nodaviridae infect vertebrates or invertebrates including insects and nematodes. Their genomes and spherule RCs bear similarities to the plant-infecting *Tombusviridae* (8, 9). The associated nodavirus-tombusvirus clade was recently substantially expanded by viruses discovered through large-scale sequencing (1, 10). Nodaviruses encode a single, highly multifunctional RNA replication factor, protein A (~1,000 aa), that contains RNA methyltransferase (MTase)/capping, membrane association, RNA-dependent RNA polymerase (RdRp), and multiple self-interaction domains (**Figure 2**). Advanced sequence comparisons show that protein A embodies three of the four domains conserved across the RNA replication proteins of the large alphavirus superfamily (11) (**Figure 2**). In keeping with such expanding parallels, findings on nodavirus RNA replication and RCs often have provided precedents relevant to many (+)RNA viruses.

Flock house virus (FHV) is a particularly well-studied nodavirus and an increasingly advanced model for (+)RNA virus genome replication and other processes (12, 13). While FHV virions are

MTase: methyltransferase

RdRp: RNA-dependent RNA polymerase

FHV: flock house virus

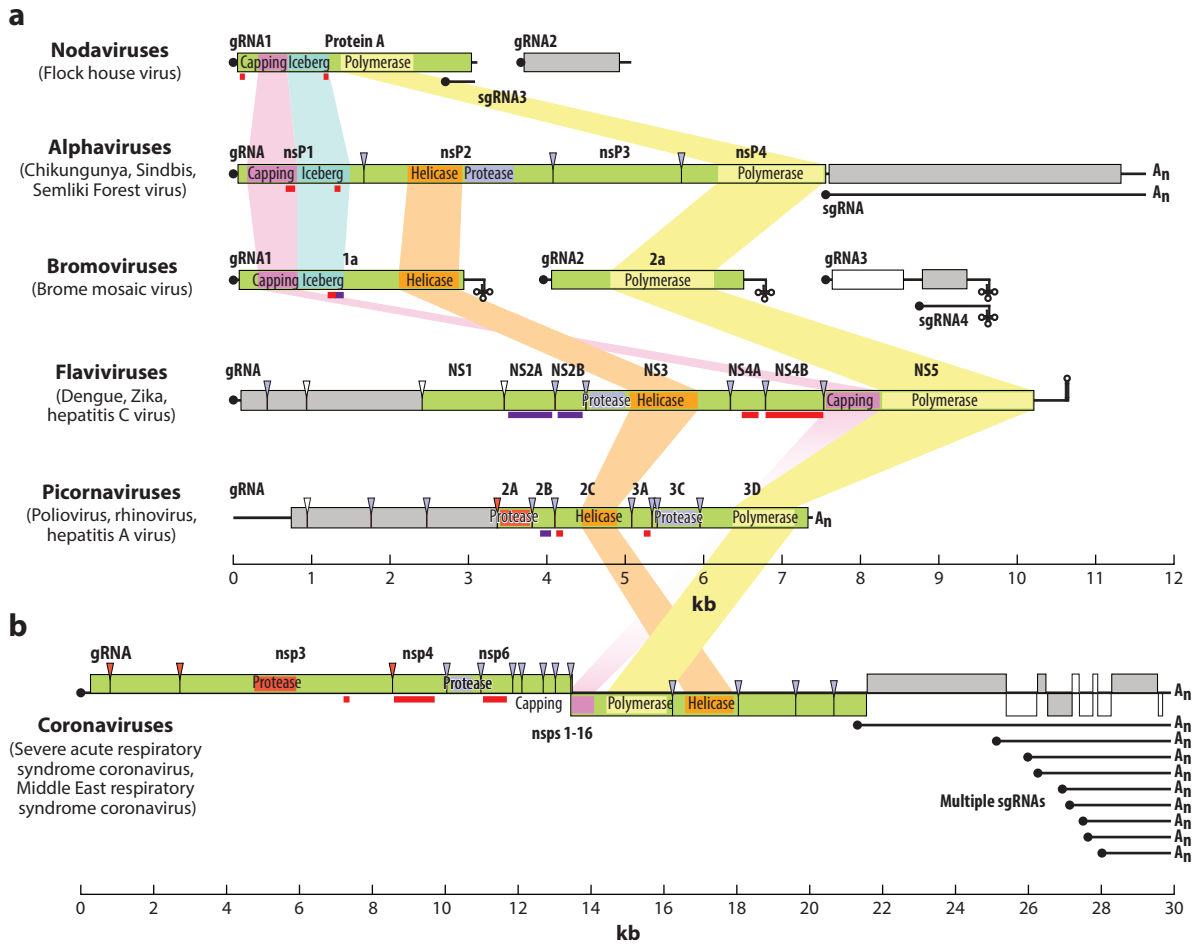

Figure 2

Genome maps representing three superfamilies and two key clades of (+)RNA viruses. Genomes of selected prototype viruses are illustrated on a common scale in panel *a*, while panel *b* uses a more condensed scale to depict the unusually large, ~30-kb genome of coronaviruses. Green boxes represent NS replicase proteins, with enzymatic domains highlighted in separate color schemes, including yellow for polymerase/RdRp, orange for NTPase/helicase, and magenta for 5′ RNA capping functions. Inverted triangles indicate protease cleavage sites color-matched with the responsible protease or left white when processed by host proteases or autocatalysis. Red bars represent membrane-associating regions. Purple bars represent viroporins. White and gray boxes represent accessory proteins and structural proteins, respectively. Black dots indicate RNA 5′ cap structures. Abbreviations: A_n, poly(A) sequences; gRNA, genomic RNA; NS, nonstructural; nsp/nsP, nonstructural protein; (+)RNA, positive-strand RNA; RdRp, RNA-dependent RNA polymerase; sgRNA, subgenomic RNA.

only infectious to insect cells, FHV RNA replication, transcription, and virion assembly have been reproduced in all eukaryotic cells tested to date, including mammalian, plant, fungal, and nematode cells (14, 15). FHV thus provides a simple, well-characterized system for RNA-dependent RNA amplification in diverse cells, which as noted above could have many practical applications.

2.1.1. Ultrastructure of nodavirus spherule RNA replication complexes. Nodavirus RNA replication occurs in spherular membrane invaginations on host mitochondria (16–20) (**Figure 3*a***). Protein A is targeted to the outer mitochondrial membrane by an N-proximal

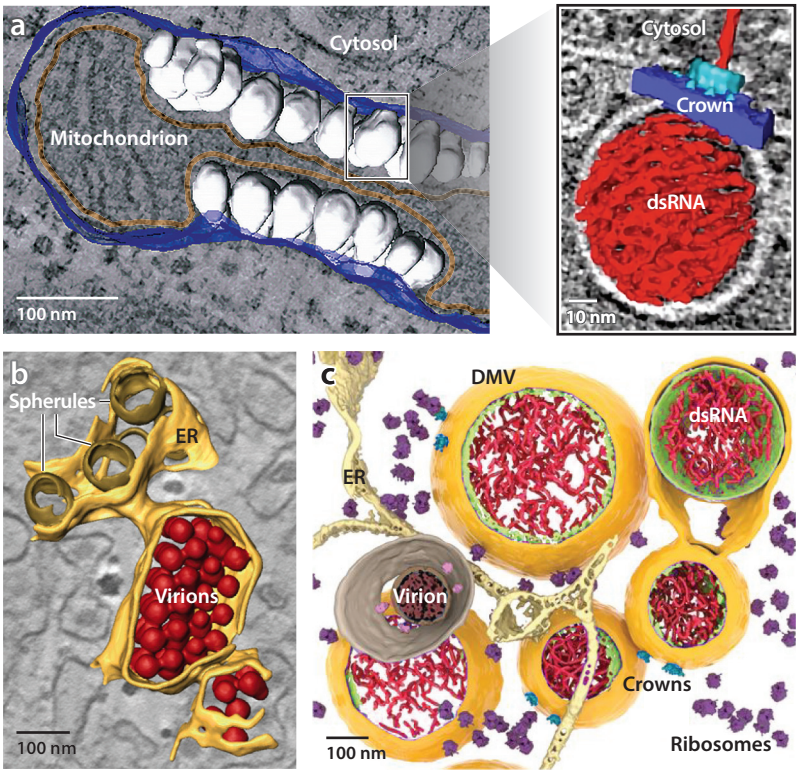

Figure 3

Three-dimensional tomographic reconstructions of RNA replication organelles induced by (*a*) flock house virus (nodavirus), (*b*) dengue virus (flavivirus), and (*c*) mouse hepatitis virus (coronavirus). The left image in panel *a* and panel *b* are derived from classical EM tomography using chemically fixed, plastic-embedded cells. The right image in panel *a* and panel *c* are from cryo-EM tomography. In panel *a*, the outer and inner mitochondrial membranes are outlined in blue and brown, respectively. In panels *b* and *c*, ER-derived membranes facing the cytosol are outlined in yellow. Red filaments represent dsRNA replication intermediates. Abbreviations: cryo-EM, cryo-electron microscopy; DMV, double-membrane vesicle; dsRNA, double-stranded RNA; EM, electron microscopy; ER, endoplasmic reticulum. Figure adapted with permission from References 17, 26, 52, and 65.

mitochondrial transmembrane domain (19) and further interacts with that membrane through sequences in its Iceberg region (11, 21, 22) (**Figure 2**). This nodavirus Iceberg region has parallels to alphavirus RNA replication proteins in predicted structure and in primary sequence position relative to flanking protein domains (**Figure 2**), and is defined as a relatively large conserved structural extension to the capping enzyme core, reminiscent of the submerged part of an iceberg (11). Protein A also recruits FHV genomic RNA templates to the outer mitochondrial membrane by interaction of its RdRp domain with *cis* signals that in FHV genomic RNA1 reside in nucleotides 68–205 (22, 23). In a process closely linked to RNA synthesis from such templates, protein A then induces invagination of the characteristic ~60- to 70-nm spherule vesicles of its RCs (24).

Classical electron microscopy (EM) tomography of FHV-infected *Drosophila* cells provided the first three-dimensional view of a (+)RNA virus RC, confirming that all RC vesicles are continuous with the outer mitochondrial membrane, with a necked connection to the cytosol (17, 25) (**Figure 3*a***, left panel). Similarly, FHV-modified mitochondria provided the first

EM: electron microscopy

three-dimensional cryo-EM analysis of (+)RNA virus RCs, revealing the RC in a near-native state and yielding striking views of the RNA and protein components of RCs, in addition to membranes (26) (**Figure 3*a***, right panel). Among other results, spherules are densely packed with coiled filaments, matching extensive biochemical evidence that they contain FHV dsRNA replication intermediates (17, 25). As with alphaviruses (27), spherule size correlates with the length of the RNA replicated, with RNA density calculations indicating that most FHV spherules contain one dsRNA (26).

2.1.2. Crown organization of viral RNA replication proteins at spherule necks. One stunning cryo-EM revelation was a 12-fold symmetric, ringed, proteinaceous complex, designated the crown, above the cytosolic side of the spherule neck (26) (**Figure 4*a***). Antibody labeling, volume calculations, and other results show that this crown consists largely if not solely of viral RNA replication protein A (21, 26). Multiple advances resolved the nodavirus crown structure to ~8.5 Å, revealing stacked rings of apical and basal lobes comprising a central turret with an interior floor and exterior legs (21) (**Figure 4*a*,*d***). The crown interacts with the outer mitochondrial membrane both at the base of the central turret and at each leg, closely controlling the shape of the RC vesicle neck. Through these 12-fold repeated interactions, the crown stabilizes the high-energy

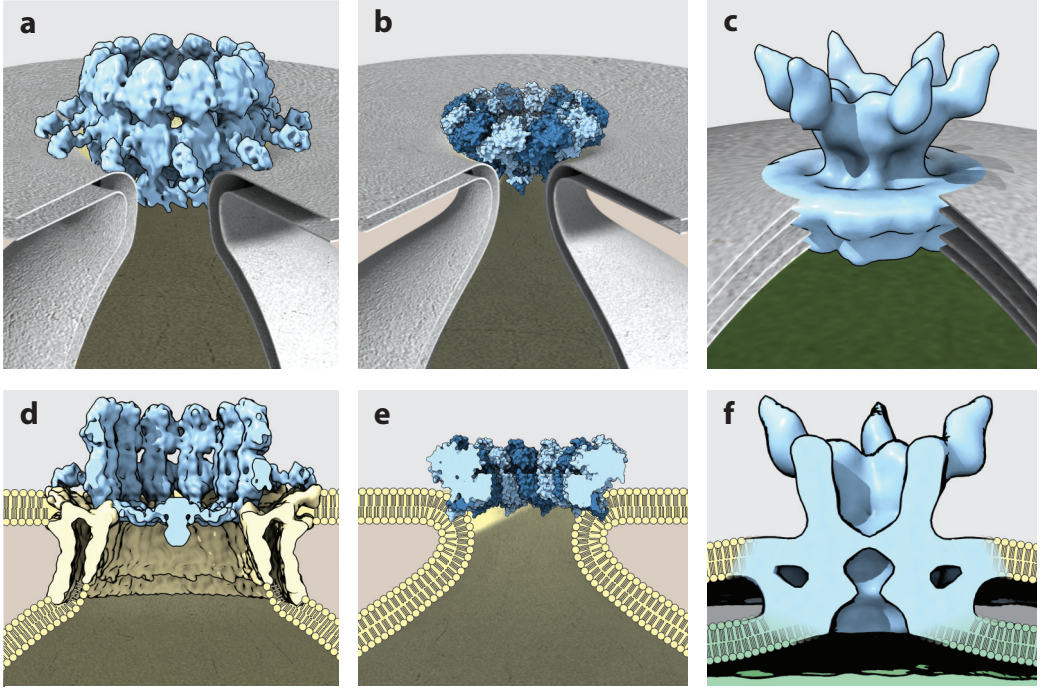

Figure 4

Detailed perspective and cross-sectional views of crown complexes of (*a*,*d*) nodavirus at 8.5 Å resolution, (*b*,*e*) alphavirus at 2.6 Å resolution, and (*c*,*f*) coronavirus at 30.5 Å resolution. Positioning of the nodavirus and coronavirus crowns in their respective membranes is as defined by cryo-EM tomographic reconstructions, while the positioning of the alphavirus crown on the membrane diagrams is interpreted from detergent interactions observed in single-particle cryo-EM reconstruction. In panels *b* and *e*, two different shades of blue are used to visualize individual nsP1 protomers. Abbreviations: cryo-EM, cryo-electron microscopy; nsP, nonstructural protein. Images are derived from electron density maps and structure coordinates associated with References 21, 41, 42, and 65 and drawn to a common scale.

spherule structure against the high membrane curvature of the neck and electrostatic repulsion of the closely packed dsRNA. Crowns frequently are the origins of filaments that likely represent nascent progeny (+)RNAs being exported into the cytosol (26). Below we outline recent findings that similar crown-like complexes of RNA replication factors are formed by alphaviruses and coronaviruses, suggesting that such features are widely conserved in (+)RNA viruses.

Mapping a genetically engineered hexahistidine (His_6) tag showed that the FHV crown apical lobe corresponds to the protein A RdRp domain (21) (**Figure 4*a***). This accessible position appears consistent with the RdRp domain's roles in RNA template recruitment (22) and potentially in negative-strand RNA [(−)RNA] synthesis, but less compatible with (+)RNA synthesis due to its distance from the vesicle-bound dsRNA template (21). Thus, (+)RNA synthesis might depend on an alternate protein A state with its RdRp domain deeper in the crown, either by bending of one or more crown apical lobes toward the vesicle or through an alternate form of protein A, as suggested by asymmetric electron-dense bodies often seen inside the crown turret (21). Further mapping of protein A's RNA capping and other domains within the crown should provide more powerful foundations for understanding and testing crown function and RC assembly and maturation.

Given these new revelations on FHV RC and crown structure, the tight linkage of FHV spherule formation to viral RNA synthesis (24) suggests that the spherule RC may be formed during (−)RNA synthesis by inflating the vesicle with the dsRNA product, like blowing up a balloon (12). A similar model was proposed among other alternatives for Semliki Forest alphavirus (27).

(−)RNA: negative-strand RNA

CHIKV: Chikungunya virus

2.2. Alphavirus-Like Superfamily

The alphavirus-like superfamily of (+)RNA viruses includes members that infect animals, insects, fungi, and plants, united by four conserved domains in their RNA replication proteins: an RNA MTase/capping domain, membrane-interactive Iceberg region (11), superfamily 1 NTPase/helicase, and RdRp (**Figure 2**). Through multiple phylogenetic interconnections, this alphavirus-like superfamily, the nodavirus-tombusvirus clade discussed above, and the flavivirus superfamily discussed below are now grouped together in phylum *Kitrinoviricota*, which represents ∼50% of (+)RNA viruses (1). This broad linkage underscores structural and functional parallels discussed here within and beyond this phylum.

Alphavirus RNA replication has best been studied for Semliki Forest virus and Sindbis virus, and increasingly for some more clinically important pathogens such as Chikungunya virus (CHIKV) (28–30). Alphaviruses encode a nonstructural polyprotein that is processed by a viral protease into nonstructural proteins (nsPs) 1–4 (**Figure 2**). nsP1 contains the RNA MTase/capping domain, the Iceberg region, and a second membrane-associating region. nsP2 has the NTPase/helicase, the protease, and a potential 2′-O-MTase. nsP3 has a macrodomain, zinc-binding domain, and hypervariable region. nsP4 contains the RdRp domain. Alphavirus RNA replication occurs in characteristic ∼60- to 70-nm spherule vesicles (31, 32) that are induced on the plasma membrane and then, to degrees varying among different alphaviruses, are endocytosed into endosomal and lysosomal compartments (33, 34).

Equivalent spherule RCs are induced by numerous plant-infecting alphavirus-like viruses (35, 36). EM studies confirmed the same spherule architecture as for alphaviruses and provided particularly detailed views of spherule necks through freeze-fracture studies with turnip yellow mosaic virus (37). Evidence for spherules as the sites of viral RNA synthesis progressively accumulated from multiple viruses. For example, autoradiography mapped incorporated ^{3}H-uridine to cytopathic vesicles bearing spherules (32), electron-dense fibrils inside spherules were identified as dsRNA—i.e., potential replication intermediates—by RNase sensitivity in low salt (38) and dsRNA-specific antibodies (39), and Br-rUTP incorporation was mapped directly to spherules by EM immunogold labeling (40). Further details of spherule formation and function were revealed

BMV: brome mosaic virus

NS: nonstructural

by brome mosaic virus (BMV) studies showing that nsP1/nsP2-like BMV 1a protein (**Figure 2**) induces spherule formation, recruits genomic (+)RNA through specific *cis*-signals to the spherule interior, and recruits nsP4-like BMV $2a^{pol}$ to synthesize first one (−)RNA retained in the spherule and then many (+)RNAs released to the cytosol (40).

Additional connections between alphavirus and nodavirus RCs emerged when cryo-EM single-particle analysis of CHIKV nsP1 revealed an nsP1 dodecamer ring (41, 42) (**Figure 4*b***). This nsP1 dodecamer has been visualized to date from exogenously expressed, detergent-solubilized nsP1, and not in an alphavirus RC. Nevertheless, the parallel 12-fold symmetry of crowns in active nodavirus RCs (**Figure 4*a***) and conservation of the capping domain, Iceberg region, and polymerase domain between alphaviruses and nodaviruses (**Figure 2**) suggests that the nsP1 dodecamer likely forms the membrane-bound base of a larger crown also incorporating nsP2, nsP3, and the nsP4 RdRp, and serves as a cytosolic export channel for progeny (+)RNAs.

One nsP1-nsP1 dodecamer interface includes amphipathic detergent-binding projections, suggesting that oligomerization and membrane binding may be coupled. As in nodavirus crowns (21), nsP1's ordered contacts with lipids (43, 44) likely generate and stabilize the curvature of the membrane neck. Additionally, the nsP1 MTase domain structure shares features with eukaryotic mRNA cap (guanine-N7) MTase Ecm1, suggesting structural and mechanistic interpretations of alphavirus 5′ RNA capping reactions involving m^7GTP methylation, m^7GMP-nsP1 complex formation, and m^7GpppRNA formation.

2.3. Flaviviruses

A third class of (+)RNA viruses forming spherule RCs are the *Flaviviridae*, which include major pathogens such as Zika, yellow fever, and dengue viruses. Flaviviruses encode a single polyprotein that is cleaved into three structural proteins and seven nonstructural (NS) RNA replication proteins (45) (**Figure 2**). NS5 contains the RdRp and RNA capping enzyme. NS3 contains an RNA helicase/NTPase and the polyprotein processing protease. NS2A, NS2B, NS4A, and NS4B are membrane-spanning proteins that induce a continuous membrane network comprising vesicle packets, convoluted membranes, and virion assembly/budding sites (46, 47).

Flavivirus-induced vesicle packets contain the viral RNA replication proteins, dsRNA, and nascent RNA and are the site of genome replication (48, 49). Three-dimensional EM tomography showed vesicle packets to comprise a bounding membrane vesicle bearing multiple spherule invaginations retaining necked openings to the cytosol (50–52) (**Figure 3*b***), confirming parallels noted in EM tomography of nodavirus spherules (17) (**Figure 3*a***). The convoluted membranes contain replicase proteins but no RNA, and they may be sites for replication factor production and storage preparatory to RC formation (52).

While most *Flaviviridae* form spherule RCs, HCV induces some closed single-membrane vesicles and more DMVs (53, 54). Further features and evolutionary implications of these DMVs are reviewed below.

3. DOUBLE-MEMBRANE VESICLE RNA REPLICATION COMPLEXES

In contrast to (+)RNA viruses forming spherule RCs, RNA replication by some (+)RNA viruses—including coronaviruses, picornaviruses, noroviruses, and hepaciviruses—is accompanied by formation of single- and double-membrane vesicles or tubules lacking membrane openings to the cytosol (55). For coronaviruses, DMVs were recently established as the major sites of viral RNA synthesis (56), while for other viruses in this category the respective relevance of single- and double-membrane compartments for RNA replication remains less clear. Below we briefly review these results and their implications.

3.1. Coronaviruses

Coronaviruses and in particular the beta-coronaviruses are important human and animal pathogens. They include the causal agents of the prior severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) epidemics (57) and the current devastating COVID-19 pandemic. Coronaviruses have ~30-kb genomes, the largest known genomes among (+)RNA viruses (**Figure 2*b***). Over 70% of these genomes encode enormous, overlapping 1a and 1ab polyproteins that are essential for RNA replication and for coronaviruses' unusual discontinuous subgenomic mRNA transcription (58). These polyproteins are processed by two virus-encoded proteases into 16 individual nonstructural proteins (nsps) providing functions in RNA replication organelle formation (nsps 3, 4, and 6) plus enzymatic functions such as protease (nsp3 and nsp5), RdRp (nsp12 and cofactors nsps 7 and 8), RNA capping (nsps 12, 14, and 16 and cofactor nsp10), helicase (nsp13), exonuclease (nsp14), and endonuclease (nsp15) (59). The structures of many nsPs have been solved (59), but how they collectively interact for RNA synthesis and export remains a question.

Proteins nsp3, 4, and 6 each have multiple membrane-association domains and induce a network of interconnected, endoplasmic reticulum (ER)-derived membrane rearrangements including complex convoluted membranes and hundreds of 200- to 300-nm DMVs (60–62) (**Figure 3*c***). While viral RNA replication proteins are observed throughout this network, dsRNA is found only in DMVs (56, 63, 64). Moreover, autoradiography of actinomycin-D-resistant ^{3}H-uridine incorporation in coronavirus-infected cells identified DMVs as the dominant if not sole sites of coronavirus RNA synthesis (56). DMVs arise ~2 h postinfection. Their outer membranes are often interconnected through neck-like channels to other DMVs and to convoluted membranes (64). Convoluted membranes arise later than DMVs and, unlike DMVs, do not harbor RNA. However, they abundantly immunolabel for coronavirus nsps and, analogous to similar flavivirus-induced structures, could be sites for replicase production, processing, and storage (64).

A long-elusive path for the potential release of viral RNA products from DMV interiors was provided when cryo-EM tomography revealed a sixfold symmetrical pore that spans both DMV membranes and contains nsp3, the largest (>1,900 aa) coronavirus RNA replication factor (65) (**Figure 4*c***). This coronavirus channel or crown parallels the crowns previously recognized on spherule RCs (**Figure 4*a*,*b***) in being a ringed complex of viral RNA replication factors that gates cytosolic release of viral RNA products and may assist in guiding their synthesis. Both the cytosolic and luminal portions of this coronavirus crown appear to interact with other viral and cellular factors (65). Potential luminal interactions with other viral RNA synthesis factors could help to coordinate synthesis and release of RNA replication and transcription products. On the crown's cytosolic side, nsp3's ubiquitin-like N terminus interacts with viral RNA and nucleocapsid protein (66–69), greatly enhancing RNA replication, likely by facilitating RNA delivery to the RC (66, 67, 70). As addressed below, in later infection these same nsp3/nucleocapsid interactions may guide encapsidation of progeny genomic RNA.

3.2. Picornaviruses, Hepaciviruses, and Noroviruses

Picornaviruses are a large family of human and animal viruses that include poliovirus; rhinoviruses A, B, and C; hepatitis A virus; and many others (71). Like flaviviruses, picornaviruses express a single polyprotein processed by virus-encoded proteases into structural and nonstructural proteins (**Figure 2*a***). The nonstructural RNA replication proteins include the 2A^{Pro} and 3C^{Pro} proteases, 2C NTPase, RNA synthesis primer 3B/VPg, and 3D^{Pol} RdRp. Domains conserved with 2C NTPase, 3B/VPg primer, 3C^{Pro}, and 3D^{Pol} are shared by a superfamily of picornavirus-like viruses spanning vertebrate, invertebrate, and plant hosts (2). Within this superfamily, the

functions of these conserved domains and RNA replication generally have been studied best in poliovirus (72).

Membrane association of picornavirus RNA replication proteins is mediated through 2B, 2C, and 3A, which are sufficient to induce DMVs (55). Picornavirus-infected cells undergo extensive reorganization of ER, Golgi, and lysosomal membranes to initially produce single-membrane-bounded tubules that are progressively converted into double-membrane or multi-lamellar structures by flattening, curving, and fusing single-membrane tubules (73). Viral RNA replication proteins, dsRNA, and, most importantly, Br-rUTP labeling of newly synthesized RNA were all associated with both single- and double-membrane structures, suggesting that the nature of active picornavirus RCs may be dynamic (73). A possible picornavirus connection to coronavirus crowns is suggested by features of viral protein 2C (**Figure 2**): Like coronavirus crown constituent nsp3, picornavirus 2C is a crucial, conserved RNA replication factor that binds membranes, helps to induce DMV formation, and forms hexamers (74–76).

As noted above, HCV differs from most spherule RC-forming *Flaviviridae* in generating some closed single-membrane vesicles, particularly early in infection, and larger numbers of ~150- to 300-nm DMVs later in infection. As with coronaviruses and picornaviruses, HCV DMVs appear linked with RNA replication because their emergence matches RNA replication kinetics, they label with antibodies to dsRNA, and they are induced by and contain HCV RNA replication factor NS5A (53, 54, 77). As considered further below, the ability of similar *Flaviviridae* genomes to give rise to either spherule RCs (dengue virus and other flaviviruses) or DMV RCs (HCV) (78) has potential implications for the structural, functional, and potentially evolutionary relation of spherules and DMVs.

Finally, similar to picornaviruses and hepaciviruses, noroviruses induce a progression from ER-linked single-membrane to double-membrane and even multi-lamellar vesicles (79). Similar structures were induced by norovirus nonstructural protein NS4 and modulated by NS1–2 and NS3. Further work is needed to define the functions of these varied compartments in norovirus replication.

4. FURTHER MECHANISTIC IMPLICATIONS

4.1. Functional Parallels and Potential Evolutionary Links Across Viruses

The RC findings summarized here reveal parallels among and beyond (+)RNA viruses, suggesting or in some cases enriching potential underlying evolutionary connections. Spherule and DMV RCs were previously viewed as disparate structures, but the presence of crown-like rings of viral RNA replication proteins serving as product RNA release channels provides unifying links between them (12, 80). As shown in **Figure 4**, the topological similarity or difference between these structures ultimately reduces to the detailed contacts of each crown with two membrane sheets: for spherules, the outer organellar membrane and inner vesicle membrane (**Figure 4*d***); for DMVs, the outer and inner vesicle membranes (**Figure 4*f***). While interpretive schematics such as **Figure 4** tend to emphasize continuity of the membranes interacting with nodaviral crowns on spherule RCs and separation of membranes interacting with coronavirus crowns on DMVs, these membranes may not be purely continuous or discontinuous in either structure. In particular, the spherule membranes interacting with nodavirus crowns are heavily disrupted by insertion of the crown's hydrophobic anchors (21), such that many lipids interact with crown proteins rather than other lipids. Similarly, for coronavirus crowns, as imaging resolution advances it will be of great interest to determine if interactions with crown proteins lead to any approach or contact between lipids in the inner and outer membranes.

Crown structures embodying such mixed lipid-lipid and lipid-protein interactions at crown-membrane interfaces might represent evolutionary intermediates linking (+)RNA viruses employing spherule and DMV RCs. As noted earlier, one such evolutionary connection appears implicit within the *Flaviviridae* because flaviviruses and hepaciviruses use highly similar suites of RNA replication genes to generate spherules and DMVs, respectively (78). Further similar connections seem likely to emerge as phylogenetic gaps are filled by accelerating virus discovery.

At a higher level, similarities have been noted between intracellular (+)RNA virus RCs and certain types of virions. Most notably, (+)RNA virus RCs parallel the cores of dsRNA virus and retrovirus virions: Through these structures, each virus replicates its genome through an mRNA intermediate that is sequestered with its polymerase in a virus-induced membrane or protein compartment for negative-strand synthesis (81).

Particularly extensive parallels exist between genome replication in (+)RNA virus RCs and dsRNA viruses. The pathways and (+)RNA/dsRNA replication intermediates (**Figure 1*a***) appear identical. Indeed, (+)RNA and dsRNA virus life cycles are differentiated primarily by which replication intermediate is packaged in virions: (+)RNA/mRNA for (+)RNA viruses, and the entire dsRNA-containing RNA replication compartment or core for dsRNA viruses (81). (+)RNA virus RC crowns also show intriguing parallels with ringed viral protein channels at the fivefold vertices of dsRNA virus cores. Cystoviruses such as dsRNA bacteriophage φ6 assemble procapsids whose fivefold axes each bear an exterior hexamer of viral NTPase P4 (82). Similar to emerging models of nodavirus RC assembly (12, 26), these P4 hexamers serve as portals for active import of (+)RNA genome templates and passive release of progeny (+)RNA transcripts (83). Coronavirus DMVs also parallel these φ6 capsids in bearing multiple hexameric crowns that are thought to serve as progeny RNA release channels and might serve as RNA template import channels (65).

Reoviruses, another family of dsRNA viruses, have cores whose fivefold axes bear pentameric turrets that serve as release channels and 5′ capping complexes for viral mRNAs (84). The viral protein A that forms nodavirus crowns also contains the viral RNA capping functions (**Figure 2**) and may also 5′ cap emerging nodavirus RNAs. Finally, paralleling the 12-fold symmetry of nodavirus crowns, herpesviruses and many DNA bacteriophage use 12-mer ringed portal protein complexes as import motors and release channels for their genomic DNA (85). Thus, (+)RNA virus crowns share features with proteins from a wide range of viruses, which might represent links with common ancestors contributing to the evolution of crowns.

4.2. Dynamic Maturational Transitions of RNA Replication Complexes

Results from many (+)RNA viruses indicate that RCs are dynamic complexes that undergo sequential maturational changes and interact with a progression of viral and host interaction partners (see also below). Alphaviruses, for example, transition their RCs from (−)RNA to (+)RNA synthesis by viral nsP2 protease processing of their nsP1234 polyprotein (28, 30) (**Figure 2**). In an early step required for RNA synthesis, nsP2 releases nsP4^{pol} from nsP1234 during or immediately after polyprotein translation. The resulting nsP123 and nsP4^{pol} complex is specialized for (−)RNA synthesis, which as with nodaviruses is linked to spherule formation (33, 86). Subsequently, (−)RNA synthesis is inhibited and (+)RNA synthesis promoted by cleavages that separate first nsP1 from nsP23, and then nsP2 from nsP3 (87). The timing of nsP1/2 cleavage appears optimized to allow conformational adjustments of nsP-nsP interactions required for subsequent steps, including nsP2/3 cleavage (88–90).

This alphavirus polyprotein processing takes on new meaning with the finding that nsP1 assembles a 12-mer ring (41, 42) (**Figure 4**) that likely serves as a base for at least early nsP123 and nsP1234 complexes. Such high copy nsP1234 multimers would have similarities to the 12-fold symmetric, multi-domain nodavirus crowns (21, 26) and intriguing implications for functional

issues such as intra- and intermolecular cleavages by nsP2 and the need to proteolytically release nsP4^{pol} for RNA synthesis.

Further indication of RC maturational changes emerged from recognition that alphavirus-like viruses, flaviviruses, and other *Kitrinoviricota* exploit cellular oxidizing functions to enable later aspects of (+)RNA synthesis including RNA 5′ capping (91). Antioxidant treatment or knockdown of specific oxidases inhibits genome replication by BMV, red clover necrotic mosaic virus, alphaviruses, and flaviviruses (92–94). Conversely, oxidant treatment in vitro or overexpression of specific oxidases in vivo enhances 5′ RNA capping by the relevant viral capping enzymes. Disulfide-linked viral RNA capping protein multimers are formed in flavi- and alpha-like virus infections, and their abundance correlates with such oxidant-dependent capping activation (92, 94, 95).

How viral RNA capping enzymes could access cellular oxidants was initially unclear because these oxidants are compartmentalized within organellar lumens, while the relevant RCs (**Figures 3** and **4**) are spherules that connect via their membrane necks and crowns to the highly reduced cytosol (96). Recent studies showed that BMV RNA replication protein 1a, whose multiple functions include RNA capping, permeabilizes the ER membranes on which BMV spherule RCs form, locally oxidizing the adjacent cytosol (94). The effects of 1a mutations and other results suggest that this oxidation induces a 1a-1a disulfide-linked complex whose conformational changes activate 1a's RNA capping functions (94). These studies also mapped 1a's membrane-permeabilizing or viroporin functions to an amphipathic alpha-helix conserved among alpha-like viruses (11). Because flavivirus NS2 and picornavirus 2B (**Figure 2**) also permeabilize intracellular membranes (97), members of all three (+)RNA virus superfamilies have RNA replication factors with viroporin activity, whose release of oxidizing potential or other small molecules from secretory or organellar lumens may regulate later steps in RNA replication (91).

4.3. Multiple RNA Replication Complex Outputs: Genomic RNAs, Subgenomic Messenger RNAs, and Recombination

The RCs of most (+)RNA viruses host multiple processes such as replicating different RNAs of multicomponent genomes, genomic and subgenomic RNA synthesis, and RNA recombination. Growing understanding of RC structure and compartmentalization enables more effective consideration of how these processes are regulated and whether individual RCs might be specialized for particular pathways or RNA templates.

To express additional proteins from internal open reading frames, many (+)RNA viruses produce subgenomic mRNAs corresponding to 5′ truncated fragments of viral genomic (+)RNA. For BMV (**Figure 2**), and by inference other alphavirus-like viruses, in vitro and in vivo results show that subgenomic mRNAs are synthesized by (+)RNA initiation at alternate internal promoters on a full-length genomic (−)RNA template (98). While many regulatory questions remain, mutual interference between BMV genomic and subgenomic RNA synthesis suggests that both pathways may proceed on a common genomic (−)RNA template in a single spherule RC (99).

For nodaviruses and coronaviruses (**Figure 2**), subgenomic RNAs are generated by terminating (−)RNA synthesis at interior sites on genomic (+)RNA templates, yielding truncated or internally deleted (−)RNAs that become independent replicons (58, 59, 100, 101). For both viruses, subgenomic (−)RNA production depends on defined RNA sequences, structures, and long-range RNA interactions (58, 101). However, the precise conditions triggering premature termination of (−)RNA synthesis and regulating relative levels of full-length genomic and partial-length subgenomic (−)RNAs remain poorly understood.

It is also unknown whether individual coronavirus or nodavirus RCs contain only one or more types of genomic or subgenomic RNA templates. For coronavirus DMVs, the presence

of multiple crowns per DMV and the apparently lower density of dsRNA intermediates relative to spherules (65) (**Figure 3*c***) suggest that individual DMVs might host multiple genomic and/or subgenomic RNA templates, with either transient or more durable associations with specific crowns. For spherule RCs, volume measurements and the presence of a single crown suggest that most spherules contain a single dsRNA replication intermediate (26, 27) (**Figure 3*a***). However, nodavirus genomic RNA2 replication both requires and subsequently suppresses synthesis of subgenomic RNA3 from genomic RNA1 (100, 101). Thus, either some of these three replicatively interdependent RNAs are produced in a single spherule or the templates and products of their replication must interact between RCs.

(+)RNA virus RNA recombination, which is crucial for short-term adaptation and long-term evolution, appears to occur primarily by template switching during RNA replication (102) and thus within RCs. Accordingly, BMV recombination requires both parental RNAs to possess signals for recruitment into RCs, and recruiting two templates into a single RC may be the limiting factor for recombination (103, 104).

4.4. Connection of Virion Assembly to RNA Replication

For many (+)RNA viruses, genome RNA synthesis shows close physical and functional links to virion assembly. For coronaviruses, for example, virion assembly and budding sites are usually closely juxtaposed with DMV RCs producing new viral genomic RNAs (56, 63, 105) (**Figure 3*c***). Coronavirus RCs and encapsidation are further linked by interaction of the cytosolic domains of RC crowns with clustered densities similar to virion ribonucleoproteins (65) and by crown protein nsp3 binding of viral nucleocapsid N (68), implying that nsp3-N interaction might initiate encapsidation of progeny RNAs as they are released from crowns (59, 65).

Flavivirus virions also assemble and bud near their genome replication sites (52) (**Figure 3*b***). For both flaviviruses and hepaciviruses, multiple RC-associated nonstructural proteins are linked to virion assembly. In particular, flavivirus NS2A and hepacivirus NS2 are key organizers of virion assembly, promoting recruitment, localization, and function of multiple viral factors at virion assembly sites jointly associated with lipid droplets and ER (106–108). In addition to NS2A, flavivirus nonstructural proteins implicated in virion assembly include at least NS2B and NS3, while implicated HCV proteins include NS2 plus NS1/P7, NS3, NS4A, and NS5A, establishing multiple potentially guiding connections between the machineries of progeny RNA and virion production (106, 108, 109).

In nodavirus infection, large arrays of unenveloped virions accumulate directly adjacent to infection-modified mitochondria bearing RCs (110), and several results illustrate functional coupling between these processes. First, nodavirus genomic RNA1 and RNA2 are only efficiently encapsidated by capsid protein translated from RNA2 synthesized by nodaviral RNA-dependent RNA replication in mitochondrial RCs, but not by capsid protein supplied in *trans* from nonreplicating mRNA transcribed from baculovirus DNA (111). Moreover, an arginine-rich motif near the capsid protein N terminus is selectively required to encapsidate genomic RNA1 but not RNA2, largely because this domain localizes capsid protein to the mitochondrial sites of RNA replication (112). Thus, for all of these viruses, virion assembly and RNA replication are linked by multiple pathways whose mechanistic principles are still incompletely understood but promise to be quite enlightening.

4.5. Opportunities for Improved Virus Control

With genome replication at the heart of (+)RNA virus life cycles, RC assembly, maturation, and function are prime targets for antiviral strategies. Because natural (+)RNA virus infections

typically start at low multiplicity, the incoming single-stranded RNA genome (**Figure 1*a***) is highly vulnerable to normal cellular RNA turnover prior to RNA replication. Thus, merely slowing RC assembly can have dramatic antiviral effects. In addition to attractive potentials for targeting viral replication factor synthesis, stability, trafficking, and assembly, RC assembly might be inhibited by targeting relevant host factors. While space does not allow general review here of the myriad host factor requirements for (+)RNA virus replication (e.g., 45, 113–115), some examples are instructive. Host pathways involved in lipid synthesis, modification, and trafficking are prominent illustrations of potential targets because most or all (+)RNA viruses depend on and recruit such functions to RC assembly sites (116, 117). Similarly, RC generation by many (+)RNA viruses requires host membrane remodeling factors such as ESCRT proteins, reticulons, and others (118–120). Because these and many other host pathways are used by numerous (+)RNA viruses, targeting well-selected factors could provide valuable broad-spectrum antiviral effects.

After RNA replication starts, eukaryotic innate immune defenses that recognize (+)RNA virus dsRNA (121) include RNA interference (120) and multiple pattern recognition receptors (122–124). As expected from their structure (**Figure 3**), (+)RNA virus RCs strongly sequester viral dsRNA from interaction with such extravesicular factors (40, 125). Innate immune responses thus might be initiated primarily from rare aberrant RCs whose mis-assembly or other defects render dsRNAs accessible. Accordingly, treatments that increase defective RC production might greatly accelerate and stimulate innate immune responses. Intriguingly, crown components SARS-CoV nsp3 and CHIKV nsP1 bind and antagonize innate immune recognition/signaling factors MDA5 (124) and cGAS-STING (126), suggesting that viral crowns might provide active as well as passive defenses against innate immunity.

5. CONCLUDING REMARKS

In just the past few years, the discovery of crowns in the RCs of several distinct viruses (21, 26, 41, 42, 65) has begun to establish these intriguing, strategically placed complexes as critical features in the replication of many if not most (+)RNA viruses. While the structure of these crowns is still being unraveled, their recognition is already providing foundations for resolving central questions of RC organization, assembly, and function that have been recognized for decades. The identification of crowns emerged from the convergence of revolutionary advances in molecular virology, cryo-EM imaging, and other areas, and these same trends promise accelerating future progress. Further structural and functional studies will undoubtedly provide important insights into multiple key mechanistic issues, many of which are posed above. In this age of heightened pandemic awareness, perhaps chief among these is how our increasing understanding of the machinery and functions of (+)RNA virus genome replication and associated processes can best be translated into more effective and ideally more broadly active antiviral control strategies. Gratifyingly, emerging results already imply that dramatic new surprises and productive opportunities for research and applications will be in store as this exciting arena advances.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We thank the Rowe Center for Research in Virology and National Institutes of Health for research support, and members of our laboratory for valuable discussions and research assistance. We are

grateful to H. Adam Steinberg for insightful contributions to figure design and generation. We regret that space constraints preclude summarizing all relevant work in this rapidly expanding area.

LITERATURE CITED

1. Wolf YI, Silas S, Wang Y, Wu S, Bocek M, et al. 2020. Doubling of the known set of RNA viruses by metagenomic analysis of an aquatic virome. *Nat. Microbiol.* 5(10):1262–70
2. Wolf YI, Kazlauskas D, Iranzo J, Lucía-Sanz A, Kuhn JH, et al. 2018. Origins and evolution of the global RNA virome. *mBio* 9(6):e02329-18
3. English JG, Olsen RHJ, Lansu K, Patel M, White K, et al. 2019. VEGAS as a platform for facile directed evolution in mammalian cells. *Cell* 178(3):748–61.e17
4. Ball P. 2021. The lightning-fast quest for COVID vaccines—and what it means for other diseases. *Nature* 589(7840):16–18
5. Abudayyeh OO, Gootenberg JS. 2021. CRISPR diagnostics. *Science* 372(6545):914–15
6. Winkle M, El-Daly SM, Fabbri M, Calin GA. 2021. Noncoding RNA therapeutics—challenges and potential solutions. *Nat. Rev. Drug Discov.* 20(8):629–51
7. Liu G, Lin Q, Jin S, Gao C. 2022. The CRISPR-Cas toolbox and gene editing technologies. *Mol. Cell* 82(2):333–47
8. Burgyan J, Rubino L, Russo M. 1996. The 5′-terminal region of a tombusvirus genome determines the origin of multivesicular bodies. *J. Gen. Virol.* 77(8):1967–74
9. Fernández de Castro I, Fernández JJ, Barajas D, Nagy PD, Risco C. 2017. Three-dimensional imaging of the intracellular assembly of a functional viral RNA replicase complex. *J. Cell Sci.* 130(1):260–68
10. Shi M, Lin X-D, Tian J-H, Chen L-J, Chen X, et al. 2016. Redefining the invertebrate RNA virosphere. *Nature* 540(7634):539–43
11. Ahola T, Karlin DG. 2015. Sequence analysis reveals a conserved extension in the capping enzyme of the alphavirus supergroup, and a homologous domain in nodaviruses. *Biol. Direct* 10:16
12. Unchwaniwala N, Zhan H, den Boon JA, Ahlquist P. 2021. Cryo-electron microscopy of nodavirus RNA replication organelles illuminates positive-strand RNA virus genome replication. *Curr. Opin. Virol.* 51:74–79
13. Venter PA, Schneemann A. 2008. Recent insights into the biology and biomedical applications of Flock House virus. *Cell. Mol. Life Sci.* 65(17):2675–87
14. Lu R, Maduro M, Li F, Li HW, Broitman-Maduro G, et al. 2005. Animal virus replication and RNAi-mediated antiviral silencing in *Caenorhabditis elegans*. *Nature* 436(7053):1040–43
15. Price BD, Rueckert RR, Ahlquist P. 1996. Complete replication of an animal virus and maintenance of expression vectors derived from it in *Saccharomyces cerevisiae*. *PNAS* 93(18):9465–70
16. Dye BT, Miller DJ, Ahlquist P. 2005. In vivo self-interaction of nodavirus RNA replicase protein A revealed by fluorescence resonance energy transfer. *J. Virol.* 79(14):8909–19
17. Kopek BG, Perkins G, Miller DJ, Ellisman MH, Ahlquist P. 2007. Three-dimensional analysis of a viral RNA replication complex reveals a virus-induced mini-organelle. *PLOS Biol.* 5(9):e220
18. Miller DJ, Schwartz MD, Ahlquist P. 2001. Flock house virus RNA replicates on outer mitochondrial membranes in *Drosophila* cells. *J. Virol.* 75(23):11664–76
19. Miller DJ, Ahlquist P. 2002. Flock house virus RNA polymerase is a transmembrane protein with amino-terminal sequences sufficient for mitochondrial localization and membrane insertion. *J. Virol.* 76(19):9856–67
20. Quirin T, Chen Y, Pietilä MK, Guo D, Ahola T. 2018. The RNA capping enzyme domain in protein A is essential for flock house virus replication. *Viruses* 10(9):483
21. Unchwaniwala N, Zhan H, Pennington J, Horswill M, den Boon JA, Ahlquist P. 2020. Subdomain cryo-EM structure of nodaviral replication protein A crown complex provides mechanistic insights into RNA genome replication. *PNAS* 117(31):18680–91
22. Van Wynsberghe PM, Chen H-R, Ahlquist P. 2007. Nodavirus RNA replication protein A induces membrane association of genomic RNA. *J. Virol.* 81(9):4633–44

23. Van Wynsberghe PM, Ahlquist P. 2009. 5′ *cis* elements direct nodavirus RNA1 recruitment to mitochondrial sites of replication complex formation. *J. Virol.* 83(7):2976–88
24. Kopek BG, Settles EW, Friesen PD, Ahlquist P. 2010. Nodavirus-induced membrane rearrangement in replication complex assembly requires replicase protein A, RNA templates, and polymerase activity. *J. Virol.* 84(24):12492–503
25. Short JR, Speir JA, Gopal R, Pankratz LM, Lanman J, Schneemann A. 2016. Role of mitochondrial membrane spherules in Flock House virus replication. *J. Virol.* 90(7):3676–83
26. Ertel KJ, Benefield D, Castaño-Diez D, Pennington JG, Horswill M, et al. 2017. Cryo-electron tomography reveals novel features of a viral RNA replication compartment. *eLife* 6:e25940
27. Kallio K, Hellström K, Balistreri G, Spuul P, Jokitalo E, Ahola T. 2013. Template RNA length determines the size of replication complex spherules for Semliki Forest virus. *J. Virol.* 87(16):9125–34
28. Ahola T, McInerney G, Merits A. 2021. Alphavirus RNA replication in vertebrate cells. *Adv. Virus Res.* 111:111–56
29. Pietilä MK, Hellström K, Ahola T. 2017. Alphavirus polymerase and RNA replication. *Virus Res.* 234:44–57
30. Rupp JC, Sokoloski KJ, Gebhart NN, Hardy RW. 2015. Alphavirus RNA synthesis and non-structural protein functions. *J. Gen. Virol.* 96(9):2483–500
31. Froshauer S, Kartenbeck J, Helenius A. 1988. Alphavirus RNA replicase is located on the cytoplasmic surface of endosomes and lysosomes. *J. Cell Biol.* 107(6):2075–86
32. Grimley PM, Berezesky IK, Friedman RM. 1968. Cytoplasmic structures associated with an arbovirus infection: loci of viral ribonucleic acid synthesis. *J. Virol.* 2(11):1326–38
33. Frolova EI, Gorchakov R, Pereboeva L, Atasheva S, Frolov I. 2010. Functional Sindbis virus replicative complexes are formed at the plasma membrane. *J. Virol.* 84(22):11679–95
34. Spuul P, Balistreri G, Kääriäinen L, Ahola T. 2010. Phosphatidylinositol 3-kinase-, actin-, and microtubule-dependent transport of Semliki Forest virus replication complexes from the plasma membrane to modified lysosomes. *J. Virol.* 84(15):7543–57
35. Hatta T, Ushiyama R. 1973. Mitochondrial vesiculation associated with cucumber green mottle mosaic virus-infected plants. *J. Gen. Virol.* 21(1):9–17
36. Kim KS. 1977. An ultrastructural study of inclusions and disease development in plant cells infected by cowpea chlorotic mottle virus. *J. Gen. Virol.* 35(3):535–43
37. Hatta T, Bullivant S, Matthews RE. 1973. Fine structure of vesicles induced in chloroplasts of Chinese cabbage leaves by infection with turnip yellow mosaic virus. *J. Gen. Virol.* 20(1):37–50
38. Hatta T, Francki RIB. 1981. Cytopathic structures associated with tonoplasts of plant cells infected with cucumber mosaic and tomato aspermy viruses. *J. Gen. Virol.* 53(2):343–46
39. Lee JY, Marshall JA, Bowden DS. 1994. Characterization of rubella virus replication complexes using antibodies to double-stranded RNA. *Virology* 200(1):307–12
40. Schwartz M, Chen J, Janda M, Sullivan M, den Boon J, Ahlquist P. 2002. A positive-strand RNA virus replication complex parallels form and function of retrovirus capsids. *Mol. Cell* 9(3):505–14
41. Jones R, Bragagnolo G, Arranz R, Reguera J. 2021. Capping pores of alphavirus nsP1 gate membranous viral replication factories. *Nature* 589(7843):615–19
42. Zhang K, Law Y-S, Law MCY, Tan YB, Wirawan M, Luo D. 2021. Structural insights into viral RNA capping and plasma membrane targeting by Chikungunya virus nonstructural protein 1. *Cell Host Microbe* 29(5):757–64.e3
43. Ahola T, Lampio A, Auvinen P, Kääriäinen L. 1999. Semliki Forest virus mRNA capping enzyme requires association with anionic membrane phospholipids for activity. *EMBO J.* 18(11):3164–72
44. Zhang N, Zhao H, Zhang L. 2019. Fatty acid synthase promotes the palmitoylation of Chikungunya virus nsP1. *J. Virol.* 93(3):e01747-18
45. Neufeldt CJ, Cortese M, Acosta EG, Bartenschlager R. 2018. Rewiring cellular networks by members of the *Flaviviridae* family. *Nat. Rev. Microbiol.* 16(3):125–42
46. Ci Y, Shi L. 2021. Compartmentalized replication organelle of flavivirus at the ER and the factors involved. *Cell. Mol. Life Sci.* 78(11):4939–54
47. Xie X, Zou J, Zhang X, Zhou Y, Routh AL, et al. 2019. Dengue NS2A protein orchestrates virus assembly. *Cell Host Microbe* 26(5):606–22.e8

48. Mackenzie JM, Jones MK, Young PR. 1996. Immunolocalization of the dengue virus nonstructural glycoprotein NS1 suggests a role in viral RNA replication. *Virology* 220(1):232–40
49. Westaway EG, Khromykh AA, Mackenzie JM. 1999. Nascent flavivirus RNA colocalized *in situ* with double-stranded RNA in stable replication complexes. *Virology* 258(1):108–17
50. Cortese M, Goellner S, Acosta EG, Neufeldt CJ, Oleksiuk O, et al. 2017. Ultrastructural characterization of Zika virus replication factories. *Cell Rep.* 18(9):2113–23
51. Rossignol ED, Peters KN, Connor JH, Bullitt E. 2017. Zika virus induced cellular remodelling. *Cell. Microbiol.* 19(8):e12740
52. Welsch S, Miller S, Romero-Brey I, Merz A, Bleck CKE, et al. 2009. Composition and three-dimensional architecture of the Dengue virus replication and assembly sites. *Cell Host Microbe* 5(4):365–75
53. Ferraris P, Blanchard E, Roingeard P. 2010. Ultrastructural and biochemical analyses of hepatitis C virus-associated host cell membranes. *J. Gen. Virol.* 91(9):2230–37
54. Romero-Brey I, Merz A, Chiramel A, Lee J-Y, Chlanda P, et al. 2012. Three-dimensional architecture and biogenesis of membrane structures associated with hepatitis C virus replication. *PLOS Pathog.* 8(12):e1003056
55. Wolff G, Melia CE, Snijder EJ, Bárcena M. 2020. Double-membrane vesicles as platforms for viral replication. *Trends Microbiol.* 28(12):1022–33
56. Snijder EJ, Limpens RWAL, de Wilde AH, de Jong AWM, Zevenhoven-Dobbe JC, et al. 2020. A unifying structural and functional model of the coronavirus replication organelle: tracking down RNA synthesis. *PLOS Biol.* 18(6):e3000715
57. Hilgenfeld R, Peiris M. 2013. From SARS to MERS: 10 years of research on highly pathogenic human coronaviruses. *Antivir. Res.* 100(1):286–95
58. Sola I, Almazán F, Zúñiga S, Enjuanes L. 2015. Continuous and discontinuous RNA synthesis in coronaviruses. *Annu. Rev. Virol.* 2:265–88
59. Malone B, Urakova N, Snijder EJ, Campbell EA. 2022. Structures and functions of coronavirus replication–transcription complexes and their relevance for SARS-CoV-2 drug design. *Nat. Rev. Mol. Cell Biol.* 23(1):21–39
60. Angelini MM, Akhlaghpour M, Neuman BW, Buchmeier MJ. 2013. Severe acute respiratory syndrome coronavirus nonstructural proteins 3, 4, and 6 induce double-membrane vesicles. *mBio* 4(4):e00524-13
61. Oudshoorn D, Rijs K, Limpens RWAL, Groen K, Koster AJ, et al. 2017. Expression and cleavage of Middle East respiratory syndrome coronavirus nsp3-4 polyprotein induce the formation of double-membrane vesicles that mimic those associated with coronaviral RNA replication. *mBio* 8(6):e01658-17
62. Oostra M, Hagemeijer MC, van Gent M, Bekker CPJ, te Lintelo EG, et al. 2008. Topology and membrane anchoring of the coronavirus replication complex: Not all hydrophobic domains of nsp3 and nsp6 are membrane spanning. *J. Virol.* 82(24):12392–405
63. Klein S, Cortese M, Winter SL, Wachsmuth-Melm M, Neufeldt CJ, et al. 2020. SARS-CoV-2 structure and replication characterized by in situ cryo-electron tomography. *Nat. Commun.* 11(1):5885
64. Knoops K, Kikkert M, van den Worm SHE, Zevenhoven-Dobbe JC, van der Meer Y, et al. 2008. SARS-coronavirus replication is supported by a reticulovesicular network of modified endoplasmic reticulum. *PLOS Biol.* 6(9):e226
65. Wolff G, Limpens RWAL, Zevenhoven-Dobbe JC, Laugks U, Zheng S, et al. 2020. A molecular pore spans the double membrane of the coronavirus replication organelle. *Science* 369(6509):1395–98
66. Hurst KR, Ye R, Goebel SJ, Jayaraman P, Masters PS. 2010. An interaction between the nucleocapsid protein and a component of the replicase-transcriptase complex is crucial for the infectivity of coronavirus genomic RNA. *J. Virol.* 84(19):10276–88
67. Koetzner CA, Hurst-Hess KR, Kuo L, Masters PS. 2021. Analysis of a crucial interaction between the coronavirus nucleocapsid protein and the major membrane-bound subunit of the viral replicase-transcriptase complex. *Virology* 567:1–14
68. Lei J, Kusov Y, Hilgenfeld R. 2018. Nsp3 of coronaviruses: structures and functions of a large multi-domain protein. *Antivir. Res.* 149:58–74
69. Serrano P, Johnson MA, Almeida MS, Horst R, Herrmann T, et al. 2007. Nuclear magnetic resonance structure of the N-terminal domain of nonstructural protein 3 from the severe acute respiratory syndrome coronavirus. *J. Virol.* 81(21):12049–60

70. Cong Y, Ulasli M, Schepers H, Mauthe M, V'kovski P, et al. 2020. Nucleocapsid protein recruitment to replication-transcription complexes plays a crucial role in coronaviral life cycle. *J. Virol.* 94(4):e01925-19
71. Zell R, Delwart E, Gorbalenya AE, Hovi T, King AMQ, et al. 2017. ICTV virus taxonomy profile: *Picornaviridae*. *J. Gen. Virol.* 98(10):2421–22
72. Lévêque N, Semler BL. 2015. A 21st century perspective of poliovirus replication. *PLOS Pathog.* 11(6):e1004825
73. Belov GA, Nair V, Hansen BT, Hoyt FH, Fischer ER, Ehrenfeld E. 2012. Complex dynamic development of poliovirus membranous replication complexes. *J. Virol.* 86(1):302–12
74. Adams P, Kandiah E, Effantin G, Steven AC, Ehrenfeld E. 2009. Poliovirus 2C protein forms homo-oligomeric structures required for ATPase activity. *J. Biol. Chem.* 284(33):22012–21
75. Papageorgiou N, Coutard B, Lantez V, Gautron E, Chauvet O, et al. 2010. The 2C putative helicase of echovirus 30 adopts a hexameric ring-shaped structure. *Acta Crystallogr. D* 66(10):1116–20
76. Sweeney TR, Cisnetto V, Bose D, Bailey M, Wilson JR, et al. 2010. Foot-and-mouth disease virus 2C is a hexameric AAA+ protein with a coordinated ATP hydrolysis mechanism. *J. Biol. Chem.* 285(32):24347–59
77. Romero-Brey I, Berger C, Kallis S, Kolovou A, Paul D, et al. 2015. NS5A domain 1 and polyprotein cleavage kinetics are critical for induction of double-membrane vesicles associated with hepatitis C virus replication. *mBio* 6(4):e00759
78. Chatel-Chaix L, Bartenschlager R. 2014. Dengue virus- and hepatitis C virus-induced replication and assembly compartments: the enemy inside—caught in the web. *J. Virol.* 88(11):5907–11
79. Doerflinger SY, Cortese M, Romero-Brey I, Menne Z, Tubiana T, et al. 2017. Membrane alterations induced by nonstructural proteins of human norovirus. *PLOS Pathog.* 13(10):e1006705
80. Unchwaniwala N, Ahlquist P. 2020. Coronavirus dons a new crown. *Science* 369(6509):1306–7
81. Ahlquist P. 2006. Parallels among positive-strand RNA viruses, reverse-transcribing viruses and double-stranded RNA viruses. *Nat. Rev. Microbiol.* 4(5):371–82
82. Poranen MM, Paatero AO, Tuma R, Bamford DH. 2001. Self-assembly of a viral molecular machine from purified protein and RNA constituents. *Mol. Cell* 7(4):845–54
83. Kainov DE, Lísal J, Bamford DH, Tuma R. 2004. Packaging motor from double-stranded RNA bacteriophage ϕ12 acts as an obligatory passive conduit during transcription. *Nucleic Acids Res.* 32(12):3515–21
84. Zhang X, Ding K, Yu X, Chang W, Sun J, Zhou ZH. 2015. *In situ* structures of the segmented genome and RNA polymerase complex inside a dsRNA virus. *Nature* 527(7579):531–34
85. Dedeo CL, Cingolani G, Teschke CM. 2019. Portal protein: the orchestrator of capsid assembly for the dsDNA tailed bacteriophages and herpesviruses. *Annu. Rev. Virol.* 6:141–60
86. Spuul P, Balistreri G, Hellström K, Golubtsov AV, Jokitalo E, Ahola T. 2011. Assembly of alphavirus replication complexes from RNA and protein components in a novel *trans*-replication system in mammalian cells. *J. Virol.* 85(10):4739–51
87. Lemm JA, Rümenapf T, Strauss EG, Strauss JH, Rice CM. 1994. Polypeptide requirements for assembly of functional Sindbis virus replication complexes: a model for the temporal regulation of minus- and plus-strand RNA synthesis. *EMBO J.* 13(12):2925–34
88. Bartholomeeusen K, Utt A, Coppens S, Rausalu K, Vereecken K, et al. 2018. A Chikungunya virus *trans*-replicase system reveals the importance of delayed nonstructural polyprotein processing for efficient replication complex formation in mosquito cells. *J. Virol.* 92(14):e00152-18
89. Lulla A, Lulla V, Merits A. 2012. Macromolecular assembly-driven processing of the 2/3 cleavage site in the alphavirus replicase polyprotein. *J. Virol.* 86(1):553–65
90. Lulla V, Karo-Astover L, Rausalu K, Saul S, Merits A, Lulla A. 2018. Timeliness of proteolytic events is prerequisite for efficient functioning of the alphaviral replicase. *J. Virol.* 92(14):e00151-18
91. Nishikiori M, Ahlquist P. 2021. Transmembrane redox regulation of genome replication functions in positive-strand RNA viruses. *Curr. Opin. Virol.* 47:25–31
92. Gullberg RC, Steel JJ, Moon SL, Soltani E, Geiss BJ. 2015. Oxidative stress influences positive strand RNA virus genome synthesis and capping. *Virology* 475:219–29
93. Hyodo K, Hashimoto K, Kuchitsu K, Suzuki N, Okuno T. 2017. Harnessing host ROS-generating machinery for the robust genome replication of a plant RNA virus. *PNAS* 114(7):E1282–90

94. Nishikiori M, Ahlquist P. 2018. Organelle luminal dependence of (+)strand RNA virus replication reveals a hidden druggable target. *Sci. Adv.* 4(1):eaap8258
95. Nishikiori M, Meshi T, Ishikawa M. 2012. Guanylylation-competent replication proteins of *Tomato mosaic virus* are disulfide-linked. *Virology* 434(1):118–28
96. Morgan B, Ezeriņa D, Amoako TNE, Riemer J, Seedorf M, Dick TP. 2013. Multiple glutathione disulfide removal pathways mediate cytosolic redox homeostasis. *Nat. Chem. Biol.* 9(2):119–25
97. Nieva JL, Madan V, Carrasco L. 2012. Viroporins: structure and biological functions. *Nat. Rev. Microbiol.* 10(8):563–74
98. French R, Ahlquist P. 1988. Characterization and engineering of sequences controlling in vivo synthesis of brome mosaic virus subgenomic RNA. *J. Virol.* 62(7):2411–20
99. Grdzelishvili VZ, Garcia-Ruiz H, Watanabe T, Ahlquist P. 2005. Mutual interference between genomic RNA replication and subgenomic mRNA transcription in brome mosaic virus. *J. Virol.* 79(3):1438–51
100. Eckerle LD, Albariño CG, Ball LA. 2003. Flock House virus subgenomic RNA3 is replicated and its replication correlates with transactivation of RNA2. *Virology* 317(1):95–108
101. Lindenbach BD, Sgro J-Y, Ahlquist P. 2002. Long-distance base pairing in flock house virus RNA1 regulates subgenomic RNA3 synthesis and RNA2 replication. *J. Virol.* 76(8):3905–19
102. Bentley K, Evans DJ. 2018. Mechanisms and consequences of positive-strand RNA virus recombination. *J. Gen. Virol.* 99(10):1345–56
103. Garcia-Ruiz H, Diaz A, Ahlquist P. 2018. Intermolecular RNA recombination occurs at different frequencies in alternate forms of brome mosaic virus RNA replication compartments. *Viruses* 10(3):131
104. Garcia-Ruiz H, Ahlquist P. 2006. Inducible yeast system for viral RNA recombination reveals requirement for an RNA replication signal on both parental RNAs. *J. Virol.* 80(17):8316–28
105. Cortese M, Lee J-Y, Cerikan B, Neufeldt CJ, Oorschot VMJ, et al. 2020. Integrative imaging reveals SARS-CoV-2-induced reshaping of subcellular morphologies. *Cell Host Microbe* 28(6):853–66.e5
106. Barnard TR, Abram QH, Lin QF, Wang AB, Sagan SM. 2021. Molecular determinants of flavivirus virion assembly. *Trends Biochem. Sci.* 46(5):378–90
107. Cloherty APM, Olmstead AD, Ribeiro CMS, Jean F. 2020. Hijacking of lipid droplets by hepatitis C, dengue and Zika viruses—from viral protein moonlighting to extracellular release. *Int. J. Mol. Sci.* 21(21):E7901
108. Jirasko V, Montserret R, Lee JY, Gouttenoire J, Moradpour D, et al. 2010. Structural and functional studies of nonstructural protein 2 of the hepatitis C virus reveal its key role as organizer of virion assembly. *PLOS Pathog.* 6(12):e1001233
109. Suzuki T. 2017. Hepatitis C virus replication. *Adv. Exp. Med. Biol.* 997:199–209
110. Lanman J, Crum J, Deerinck TJ, Gaietta GM, Schneemann A, et al. 2008. Visualizing flock house virus infection in *Drosophila* cells with correlated fluorescence and electron microscopy. *J. Struct. Biol.* 161(3):439–46
111. Venter PA, Krishna NK, Schneemann A. 2005. Capsid protein synthesis from replicating RNA directs specific packaging of the genome of a multipartite, positive-strand RNA virus. *J. Virol.* 79(10):6239–48
112. Venter PA, Marshall D, Schneemann A. 2009. Dual roles for an arginine-rich motif in specific genome recognition and localization of viral coat protein to RNA replication sites in Flock House virus-infected cells. *J. Virol.* 83(7):2872–82
113. Kushner DB, Lindenbach BD, Grdzelishvili VZ, Noueiry AO, Paul SM, Ahlquist P. 2003. Systematic, genome-wide identification of host genes affecting replication of a positive-strand RNA virus. *PNAS* 100(26):15764–69
114. Ramage H, Cherry S. 2015. Virus-host interactions: from unbiased genetic screens to function. *Annu. Rev. Virol.* 2:497–524
115. Baggen J, Vanstreels E, Jansen S, Daelemans D. 2021. Cellular host factors for SARS-CoV-2 infection. *Nat. Microbiol.* 6(10):1219–32
116. Zhang Z, He G, Filipowicz NA, Randall G, Belov GA, et al. 2019. Host lipids in positive-strand RNA virus genome replication. *Front. Microbiol.* 10:286
117. Ketter E, Randall G. 2019. Virus impact on lipids and membranes. *Annu. Rev. Virol.* 6:319–40
118. Diaz A, Ahlquist P. 2012. Role of host reticulon proteins in rearranging membranes for positive-strand RNA virus replication. *Curr. Opin. Microbiol.* 15(4):519–24

119. Morita E, Suzuki Y. 2021. Membrane-associated flavivirus replication complex—its organization and regulation. *Viruses* 13(6):1060
120. Nagy PD, Feng Z. 2021. Tombusviruses orchestrate the host endomembrane system to create elaborate membranous replication organelles. *Curr. Opin. Virol.* 48:30–41
121. Feng Q, Hato SV, Langereis MA, Zoll J, Virgen-Slane R, et al. 2012. MDA5 detects the double-stranded RNA replicative form in picornavirus-infected cells. *Cell Rep.* 2(5):1187–96
122. Guo Z, Li Y, Ding S-W. 2019. Small RNA-based antimicrobial immunity. *Nat. Rev. Immunol.* 19(1):31–44
123. Hopfner K-P, Hornung V. 2020. Molecular mechanisms and cellular functions of cGAS–STING signalling. *Nat. Rev. Mol. Cell Biol.* 21(9):501–21
124. Liu G, Lee J-H, Parker ZM, Acharya D, Chiang JJ, et al. 2021. ISG15-dependent activation of the sensor MDA5 is antagonized by the SARS-CoV-2 papain-like protease to evade host innate immunity. *Nat. Microbiol.* 6(4):467–78
125. Du X, Zhang Y, Zou J, Yuan Z, Yi Z. 2018. Replicase-mediated shielding of the poliovirus replicative double-stranded RNA to avoid recognition by MDA5. *J. Gen. Virol.* 99(9):1199–209
126. Webb LG, Veloz J, Pintado-Silva J, Zhu T, Rangel MV, et al. 2020. Chikungunya virus antagonizes cGAS-STING mediated type-I interferon responses by degrading cGAS. *PLOS Pathog.* 16(10):e1008999

Annual Review
of Virology

Volume 9, 2022

Contents

Genome Replication, Regulation of Gene Expression, and Biosynthesis

Virus Cell Biology

Pathogenesis

Immunity

Vaccines

Errata

An online log of corrections to *Annual Review of Virology* articles may be found at http://www.annualreviews.org/errata/virology