Cytoplasmic Viral Replication Complexes

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Many viruses that replicate in the cytoplasm compartmentalize their genome replication and transcription in organelle-like structures that enhance replication efficiency and protection from host defenses. In particular, recent studies with diverse positive-strand RNA viruses have further elucidated the ultrastructure of membrane-bound RNA replication complexes and how these complexes function in close coordination with virion assembly and budding. The structure, function, and assembly of some positive-strand RNA virus replication complexes have parallels and potential evolutionary links with the replicative cores of double-strand RNA virus and retrovirus virions and more general similarities with the replication factories of cytoplasmic DNA viruses.

Introduction

Whereas eukaryotic cells sequester and organize their genome replication and transcription in the nucleus, many RNA and some DNA viruses carry out viral genome replication and transcription in the cytoplasm. To establish efficient genome replication and shield it from host defenses, including crucial intrinsic and innate defenses, many or most of these cytoplasmically replicating viruses organize their genome replication and transcription in organelle-like compartments (Novoa et al., 2005). These replication compartments or factories often are associated with the sites of subsequent stages in the viral replication cycle, including particle formation and virus budding.

Recently, substantial advances have been made in characterizing the cytoplasmic replication compartments of positive-strand RNA viruses. Positive-strand RNA viruses package their genomes as messenger sense, single-stranded RNA and replicate those genomes solely through RNA intermediates. For a diverse set of positive-strand RNA viruses, three-dimensional, high-resolution imaging by electron microscope (EM) tomography, in combination with other complementary approaches, has revealed critical aspects of the structure and organization of membrane-bound RNA replication compartments and their close spatial and functional relationships with virus translation and virion assembly and budding sites. This review summarizes selected recent findings in this area and their relation to other RNA and DNA viruses, including implications for potential evolutionary relationships between the genome replication processes of at least some positive-strand RNA viruses and those of double-strand (ds)RNA and reverse-transcribing viruses. We regret that space limitations prevent highlighting all of the valuable contributions to these fields (Mackenzie, 2005; Miller and Krijnse-Locker, 2008; Novoa et al., 2005; Salonen et al., 2005).

Positive-Strand RNA Virus Replication Complexes

Positive-strand RNA viruses encompass more than one-third of known virus genera (ICTV, 2005) and include many medically and practically important human, animal, and plant pathogens. At the outset of infection, after their initial delivery to the cytoplasm, positive-strand RNA virus genomes are used as templates for viral protein synthesis. Among the first viral proteins to accumulate are RNA replication proteins that redirect the viral genome from functioning as an mRNA to serving as a template for synthesizing complementary negative-strand RNA, which then becomes the template for new positive-strand genomic RNAs and subgenomic mRNAs.

Pioneering ultrastructural and other studies with a variety of positive-strand RNA viruses established that viral RNA synthesis was associated with membranes and, moreover, with virus-specific membrane rearrangements such as single- and double-membrane vesicles and invaginations (e.g., Bienz et al., 1983, 1987; Froshauer et al., 1988; Grimley et al., 1968; Hatta et al., 1973; Russo and Martelli, 1972). Subsequent work confirmed that RNA replication by all positive-strand RNA viruses studied to date was linked to virus-induced, often extensive rearrangements of specific intracellular membranes (Denison, 2008; Mackenzie, 2005; Salonen et al., 2005). Below, we summarize recent findings with a diverse set of positive-strand RNA viruses that reveal new understandings of the structure, function, and assembly of these complexes; their roles in coordinating successive steps in RNA replication and beyond; their relation to dsRNA and DNA virus factories; and their evolutionary implications.

Picornaviruses

Picornaviruses are a large family of human and animal viruses whose best-studied member is poliovirus (PV) (Racaniello, 2007). The PV genome expresses a single polyprotein that is processed by viral proteases into functional intermediate precursors and fully cleaved end products. More than half of the genome encodes RNA replication factors, including the RNA-dependent RNA polymerase 3D, the 3B/VPg protein primer for RNA synthesis, and the 2C NTPase. In addition, the 2B, 2C and 3A proteins interact with each other and the other replication proteins (Yin et al., 2007) and mediate membrane association of these RNA replication proteins (Fujita et al., 2007; Teterina et al., 1997, 2006).

Interaction with the PV RNA replication proteins extensively reorganizes endoplasmic reticulum (ER), Golgi, and lysosomal
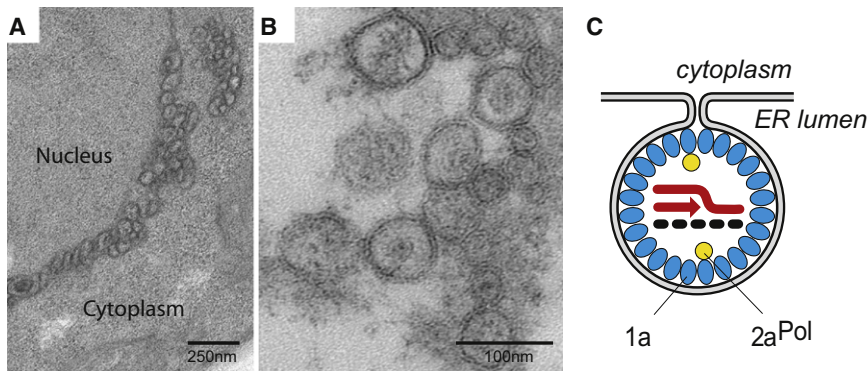
Figure 1. BMV-Induced Perinuclear ER RNA Replication Vesicles and Model of the BMV RNA Replication Complex
(A and B) (A) and (B) show low- and high-magnification EM images, respectively, of BMV-induced spherular vesicles invaginated into the perinuclear ER membrane (adapted from Schwartz et al., 2002).
(C) Diagram of the vesicular BMV RNA replication complex, illustrating the many copies of the membrane-bound, self-interacting 1a protein (blue) that induce vesicle formation, the ~20-fold fewer copies of 2a polymerase (yellow), and replicative intermediate RNAs, including positive-strand RNA (red arrows) and negative-strand RNA (black dashed arrow).

membranes into 50–400 nm single- and double-membrane-bound vesicles (Bienz et al., 1990; Cho et al., 1994; Egger et al., 2000; Schlegel et al., 1996). When these membrane-associated replication complexes are extracted from infected cells, the vesicles adopt a rosette-like appearance (Egger and Bienz, 2002). The viral replication proteins localize to the rosette center on the exposed surface of the double-vesicle membrane (Bienz et al., 1990). PV-induced membrane vesicles have been linked to COPII-dependent vesicle trafficking (Rust et al., 2001) and to activation of cellular Arf GTPases that modulate membrane trafficking (Belov et al., 2007). Recent studies further showed that viral modulation of Arf GTPase function locally enriches the membranes targeted by picornaviruses and flaviviruses (see also below) in phosphatidylinositol-4 phosphate, promoting RNA replication, potentially by facilitating recruitment of relevant viral and perhaps cellular factors and by modulating membrane curvature (Hsu et al., 2010).

The double-membrane vesicles associated with PV infection resemble double-membrane structures generated during autophagy (Jackson et al., 2005; Schlegel et al., 1996; Suhy et al., 2000; Taylor and Kirkegaard, 2008). Based on this and other work, evidence is accumulating that picornaviruses and certain other viruses induce and subvert the host autophagy pathway to support their replication, progeny virus export, or both (Dreux and Chisari, 2010; Jackson et al., 2005; Kirkegaard, 2009; Taylor and Kirkegaard, 2008).

Bromoviruses

Brome mosaic virus (BMV), a member of the alphavirus-like superfamily, conducts its RNA replication on perinuclear ER membranes (Restrepo-Hartwig and Ahlquist, 1996) in ~60 nm vesicular invaginations similar to those induced on other target membranes by many viruses in and beyond the alphavirus-like superfamily (Ahlquist, 2006; Schwartz et al., 2002) (Figure 1). BMV encodes two large RNA replication proteins: $2a^{pol}$, the viral RNA-dependent RNA polymerase, and 1a, a multifunctional protein with 5′ RNA capping and RNA NTPase/helicase domains (Ahola and Ahlquist, 1999; Ahola et al., 2000; Kong et al., 1999; Wang et al., 2005).

In addition to direct roles in RNA synthesis, 1a is the master organizer of RNA replication complex assembly. 1a directs ER membrane association (den Boon et al., 2001; Restrepo-Hartwig and Ahlquist, 1999) and, even in the absence of other viral factors, induces ER membrane invagination to form replication vesicles (Schwartz et al., 2002) (Figure 1). When present, BMV genomic RNA replication templates and $2a^{pol}$ are recruited by 1a to the ER (Chen et al., 2001; Janda and Ahlquist, 1998; Schwartz et al., 2002). 1a recruits $2a^{pol}$ through interaction of 1a's C terminus with an N-proximal region preceding $2a^{pol}$'s polymerase domain (Chen and Ahlquist, 2000; Kao and Ahlquist, 1992). Recent results indicate that $2a^{pol}$ recruitment occurs prior to and is inhibited by 1a's induction of replication vesicles (Liu et al., 2009). By contrast, 1a recruitment of viral genomic RNA templates is closely linked to replication vesicle formation (Liu et al., 2009). Template recognition and recruitment is mediated by conserved RNA sequence elements (Baumstark and Ahlquist, 2001; Chen et al., 2001; Sullivan and Ahlquist, 1999) and requires an active 1a NTPase/helicase domain, apparently to translocate the RNA into preformed vesicles (Wang et al., 2005). In these sites, both the initial positive-strand template RNA and all subsequent negative-strand RNAs are strongly protected from nuclease and presumably other cytoplasmic factors (Schwartz et al., 2002; Sullivan and Ahlquist, 1999).

EM and quantitative biochemical analyses show that a single ~60 nm BMV replication vesicle contains one or a few positive- and negative-strand RNA molecules, ~10–20 $2a^{pol}$ proteins, and ~200–400 1a proteins (Schwartz et al., 2002). This level of 1a is sufficient to coat the interior of the replication vesicle. Accordingly, because 1a is strongly membrane associated and self-interacts through multiple regions (Kao and Ahlquist, 1992; O'Reilly et al., 1995), BMV replication compartments have been proposed to contain a 1a protein shell lining the vesicle interior, suggesting a simple explanation for 1a's ability to form these compartments (Figure 1C).

Nodaviruses

Additional support for a protein shell-supported replication complex model came from similar observations for the replication complex of Flock House virus (FHV), the best-studied member of the animal nodaviruses (Venter and Schneemann, 2008). FHV encodes a single, highly multifunctional RNA replication protein, protein A. Protein A contains an N-terminal transmembrane domain targeting outer mitochondrial membranes (Miller and Ahlquist, 2002), an RNA-dependent RNA polymerase domain, and regions that direct self-interaction (Dye et al., 2005) and specific recognition and recruiting of FHV genomic RNAs to mitochondrial membranes (Van Wynsberghe and Ahlquist, 2009; Van Wynsberghe et al., 2007).

Conventional two-dimensional thin section transmission EM and three-dimensional imaging by EM tomography show that
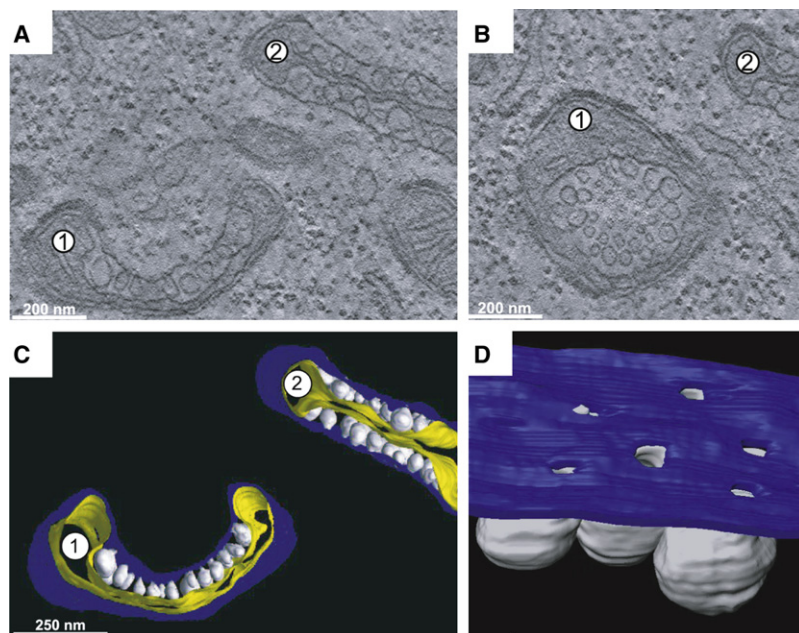
Figure 2. FHV-Induced RNA Replication Vesicles on Outer Mitochondrial Membranes
(A and B) (A) and (B) show two of a series of multiple EM tomographic images from different planes of a single thick section of FHV-modified mitochondria in an infected *Drosophila* cell. Labels 1 and 2 refer to the same mitochondria in both panels.
(C) Three-dimensional reconstruction by EM tomography of the mitochondria in (A), showing outer (blue) and inner (yellow) mitochondrial membranes and FHV-induced replication vesicles (white).
(D) Angled view of the side and top of RNA replication vesicles showing open neck-like connections with the cytoplasm (adapted from Kopek et al., 2007).

FHV induces ~50 nm vesicular invaginations between the inner and outer mitochondrial membranes (Figure 2). The interiors of these vesicles are the sites where protein A and newly synthesized FHV RNAs accumulate (Kopek et al., 2007). Stoichiometry analyses showed that each FHV replication vesicle contains ~100 copies of protein A and one or two genome RNA replication intermediates. These results are consistent with a model for the FHV replication complex very similar to that for BMV (Figure 1C), with a continuous shell of self-interacting, transmembrane protein A lining the interior of the FHV-induced mitochondrial membrane vesicle. Furthermore, tomographic imaging revealed that every FHV-induced vesicle remains attached to the outer mitochondrial membrane by a ~10 nm neck-like connection to the cytoplasm (Kopek et al., 2007) (Figure 2D).

Coronaviruses
Whereas BMV and FHV present examples of RNA replication complexes in simple vesicular membrane invaginations, members of the coronavirus and arterivirus families within the order *Nidovirales* induce more complicated mixtures of convoluted membrane rearrangements and large double-membrane vesicles (Gosert et al., 2002; Pedersen et al., 1999; Snijder et al., 2001, 2006). Among the best studied are the RNA replication structures in severe acute respiratory syndrome (SARS) coronavirus-infected cells. EM tomography studies of SARS virus-infected cells have revealed that the different membrane structures represent a single network of interconnected ER-derived membranes (Knoops et al., 2008; Knoops et al., 2010) (Figure 3).

The 5′ two-thirds of the ~30 kb genome coronavirus genome, the largest among positive-strand RNA viruses, encode polyprotein precursors that are processed into 15 or 16 RNA replicase subunits (Snijder et al., 2003; Thiel et al., 2003; Ziebuhr et al., 2000) that localize to the virus-induced membrane structures

(Knoops et al., 2008). When appropriately assayed, membrane extracts from SARS coronavirus-infected cells synthesize the typical nested set of coronavirus genomic and subgenomic RNAs. Such in vitro activity is RNase and protease resistant but detergent sensitive, indicating that the membranes provide a protective environment for RNA replication (van Hemert et al., 2008b). Similar observations were made with membrane extracts from cells infected with the distantly related arterivirus EAV (van Hemert et al., 2008a), which, in electron tomography studies, was recently found to contain a similar network of interconnected single- and double-membrane structures (K. Knoops and E.J. Snijder, personal communication).

In keeping with these results, dsRNA, the presumptive RNA replication intermediate, predominantly localizes to the interiors of the large, 200–300 nm diameter double-membrane vesicles in coronavirus-infected cells (Knoops et al., 2008). Nevertheless, it is not yet established that these vesicle interiors represent the actual sites of RNA synthesis. The outer membranes of the double-membrane vesicles are interconnected through ~8 nm tubules, but no connections between the vesicle interiors and the cytosol have yet been visualized (Knoops et al., 2008). It thus remains uncertain how ribonucleotides and product RNAs would be exchanged with the cytosol if RNA synthesis occurs inside of these double-membrane vesicles. One possible solution is that the coronavirus replication complex might use a protein channel as the equivalent of the neck-like openings in the BMV and FHV replication spherules (Knoops et al., 2008). Three of the 16 SARS RNA replication proteins have integral membrane-spanning domains (Kanjanahaluethai et al., 2007; Oostra et al., 2008) and, in principle, could support the formation of proteinaceous membrane pores to the cytoplasm. Current EM tomography images do not provide sufficient resolution to visualize or rule out the presence of such channels.

Alternatively or in addition, coronavirus RNA synthesis might occur in the convoluted single-membrane structures that adjoin and interconnect with the double-membrane vesicles. These convoluted membranes appear to be the major accumulation sites of the viral replicase subunits and encompass many spaces or compartments with open connections to the cytoplasm (Knoops et al., 2008). Later stages in the maturation of coronavirus-induced membrane rearrangements appear to involve
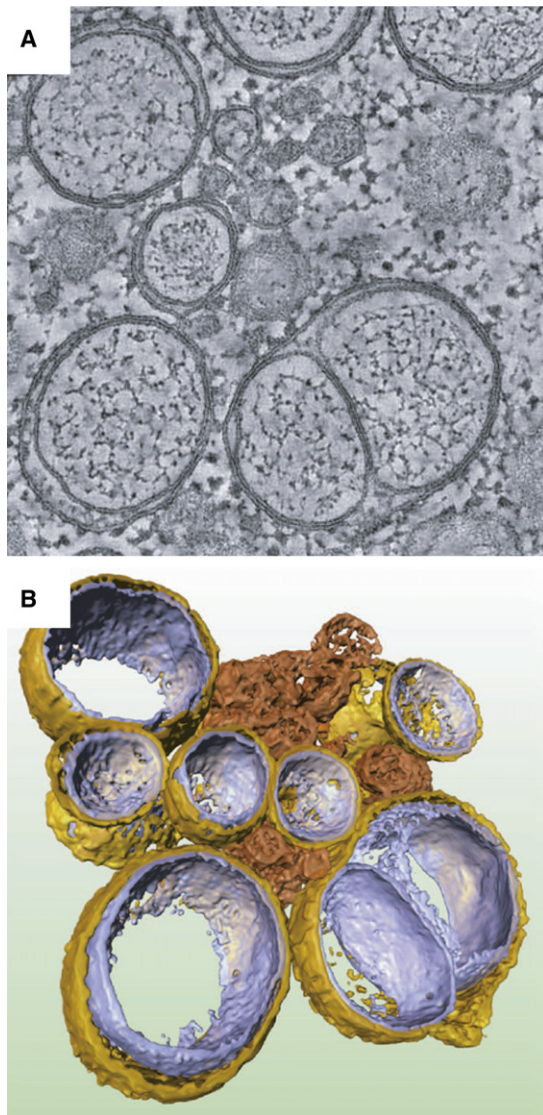
Figure 3. EM Tomographic Three-Dimensional Reconstruction of SARS Coronavirus-Induced, ER-Derived Double-Membrane Vesicles
(A and B) Two-dimensional EM sectional view (A) and three-dimensional tomographic reconstruction (B) of SARS coronavirus-induced double-membrane vesicles (yellow/blue) and convoluted membrane structures (brown) (adapted from Knoops et al., 2008).

membrane fusion events, suggesting that similar earlier fusions might allow generating the double-membrane vesicles from the interconnected convoluted membranes (Knoops et al., 2008; E.J. Snijder and M. Kikkert, personal communication). If so, the double-membrane vesicles may represent repositories that sequester dsRNAs and perhaps other by-products produced by RNA replication in the convoluted membranes.

Such possible conversion of convoluted membrane replication sites into double-membrane vesicles is reminiscent of some features of BMV RNA replication compartments. By increasing or decreasing the level of BMV 2a[pol], bromovirus replication compartments can be interconverted between layered membranes with similarities to coronavirus-induced convoluted

membranes and the vesicular invaginations normally associated with bromovirus infection (see above), which, in appropriate EM sections, appear as complexes of double-membrane vesicles (Schwartz et al., 2004).

Flaviviruses
Similarly complex replication-associated membrane structures are induced by the *Flaviviridae*, which include clinically important members such as hepatitis C virus (HCV), yellow fever virus and Dengue virus (DENV). Like poliovirus, the flavivirus genomic RNA encodes a single polyprotein that is cleaved into virion proteins and RNA replication proteins (Bartenschlager and Miller, 2008; Lindenbach and Rice, 2003; Lindenbach et al., 2007). Three of the replication proteins contain membrane-spanning domains (Mackenzie et al., 1998; Miller et al., 2006, 2007) and are responsible for inducing several distinct ER-derived membrane structures: vesicle packets, convoluted membranes, and membranes associated with progeny virus assembly (Grief et al., 1997; Mackenzie et al., 1996; Welsch et al., 2009). Recent three-dimensional EM tomography showed that the different flavivirus membrane structures are all part of a single continuous ER-derived membrane network resembling the coronavirus membrane rearrangements (Welsch et al., 2009) (Figure 4). The flavivirus-induced convoluted membrane rearrangements do not contain detectable dsRNA and have been proposed to be the sites where replication proteins accumulate, are cleaved, and are stored for further use in replication complex assembly (Mackenzie et al., 1996; Welsch et al., 2009).

The flavivirus vesicle packets consist of an outer bounding membrane surrounding a series of inner, ~90 nm vesicles (Figure 4) that contain most of the replication proteins, dsRNA, and nascent RNA (Mackenzie et al., 1996). Thus, these inner vesicles are the likely sites of genome replication. Intriguingly, these vesicle packets show features that bridge the coronavirus double-membraned vesicles and the invaginated vesicular RNA replication compartments induced by bromoviruses and nodaviruses. Like the coronavirus RNA replication compartments, in some planes of sectioning, the flavivirus vesicle packet interiors appear separated from the cytoplasm by two membranes (Figure 4). However, three-dimensional EM tomography revealed that the inner 90 nm vesicles are invaginations of the outer bounding membrane and, like BMV and FHV replication vesicles, bear necked connections to the cytoplasm (Welsch et al., 2009). This topological equivalence of flavivirus and nodavirus replication compartments had been proposed earlier (Ahlquist, 2006; Kopek et al., 2007), based on EM tomography analysis showing that certain cross sections through FHV-modified mitochondria bear remarkable resemblance to flavivirus vesicle packets (Figure 2B).

Coordination of the Viral Replication Cycle
Many viruses coordinate their genome replication with subsequent steps of producing progeny virions. DENV and SARS virus package their virion capsids in membrane envelopes, and for both viruses, EM tomography revealed that some or all virion assembly and budding steps occur within or in close proximity to the same continuous membrane networks that support genome replication (Knoops et al., 2008; Welsch et al., 2009) (Figure 4).

For DENV, in some cases, virion particle formation was observed at sites directly apposed to the open necks of the
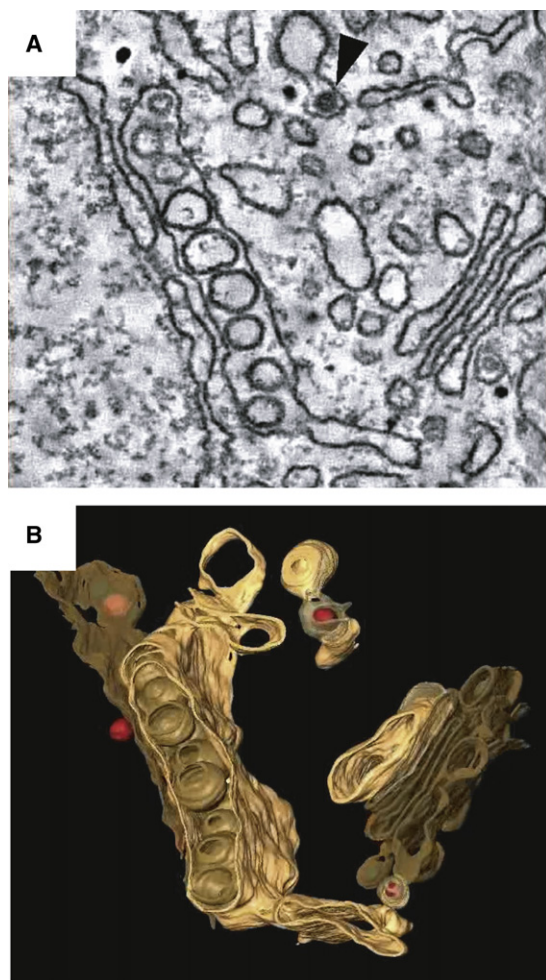
Figure 4. EM Tomographic Three-Dimensional Reconstruction of Dengue Virus-Induced, ER-Derived Vesicle Packets

(A and B) Two-dimensional EM sectional view (A) and three-dimensional tomographic reconstruction (B) of DENV-induced vesicle packets. Virion particles associated with sites of virus assembly and budding are indicated with a black arrowhead in (A) and in red in (B) (adapted from Welsch et al., 2009).

between genome replication and virion assembly exist for these viruses also. PV, BMV, and FHV virion formation all require actively replicating genomic RNA (Annamalai and Rao, 2005, 2006; Nugent et al., 1999; Venter et al., 2005). Additional EM tomography results have shown substantial FHV virion accumulation close to mitochondria with FHV genome replication complexes (Lanman et al., 2008).

Parallels with Double-Strand RNA Virus and Retrovirus Virions

Beyond close coordination of viral genome replication and virion assembly, for at least some positive-strand RNA viruses, the membrane-associated RNA replication complexes themselves show general parallels with virion assembly and structure and particular parallels with the replicative cores of dsRNA virus and reverse-transcribing virus virions (Ahlquist, 2006) (Figure 5).

As an example of dsRNA viruses, members of the well-studied *Reoviridae* family carry out most of their replication steps in cytoplasmic "viroplasms" or virus factories that, though not membrane bound, nevertheless concentrate virion assembly, RNA replication, and other steps in a defined space (Patton et al., 2006). Such dsRNA viruses encapsidate their genomic dsRNAs, together with viral polymerases, in a virion core that is active in RNA synthesis in much the same way as positive-strand RNA virus replication complexes (Figure 5A). For reoviruses, the virion core shell consists of 120 copies of viral protein λ1 and contains 60 copies of the λ2 RNA-capping protein and 12 copies of the λ3 polymerase (Reinisch et al., 2000; Zhang et al., 2003). BMV 1a and FHV protein A thus resemble λ1 as high copy number structural components of the RNA synthesis complex. 1a further resembles λ1 and λ2 in having NTPase/helicase and RNA-capping domains, respectively, whereas BMV 2a^{pol} resembles λ3 in having a polymerase domain and interacting with its cognate NTPase/helicase domain (Ahlquist, 2006) (Figures 5A and 5B). BMV 1a's roles in replication complex assembly show further similarities with reovirus protein μNS, a multifunctional protein that coordinates the recruitment and assembly of additional reovirus proteins and forms the matrix of the viroplasms within which reovirus replication and virion assembly occur (Arnold et al., 2008; Miller et al., 2010).

Additional parallels exist with retrovirus virion assembly (Bieniasz, 2009; Waheed and Freed, 2009) (Figure 5C). When the functions of the cellular ESCRT/multivesicular body sorting pathway are inhibited, e.g., retrovirus virions fail to complete their budding (Morita and Sundquist, 2004). When so arrested, retroviruses remain attached to the plasma membrane by neck-like membrane stalks that are strikingly similar to the necked membrane connections that the spherular RNA replication vesicles of FHV, BMV, alphaviruses, and many other positive-strand RNA viruses maintain to the cytoplasm. Moreover, multiple functions of the major retrovirus capsid protein Gag in virion assembly parallel roles of BMV 1a in replication complex spherule formation (Figures 5B and 5C). These similarities include targeting and defining the membrane site of virion/replication complex assembly, binding the target membrane cytoplasmic face as a peripheral membrane protein, inducing target membrane invagination, self-interacting in large numbers within the resulting vesicle, directing viral RNA templates and viral

replication vesicles (Welsch et al., 2009). Moreover, for DENV and the related hepatitis C virus, viral capsid proteins accumulate on the surface of characteristic lipid droplets (McLauchlan, 2009; Ogawa et al., 2009; Samsa et al., 2009). Like the membrane structures of the viral replication complex, these lipid droplets originate from the ER, and their abundance in the cell is directly linked to virus infection and replication (Samsa et al., 2009).

New coronavirus virions bud into ER-Golgi intermediate compartments, but early in infection, the viral nucleocapsid protein can also be detected at the double-membrane vesicle sites of viral replication (Stertz et al., 2007). Some EM tomography images of late stages of SARS virus infection showed merged replication and budding compartments (Knoops et al., 2008).

PV, BMV, and FHV, unlike flavi- and coronaviruses, are nonenveloped "naked" viruses and do not rely on membranes for budding. Nevertheless, strong spatial and functional links
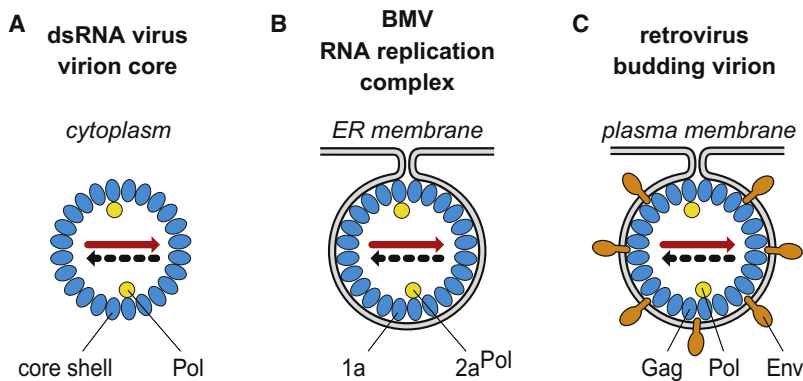
Figure 5. Parallels between Positive-Strand RNA Virus, dsRNA Virus, and Retrovirus Genome Replication
(A–C) Schematic representations of (A) the cytoplasmic replicative core of a dsRNA virus, (B) the invaginated, ER membrane-associated RNA replication complex of brome mosaic virus, and (C) a retrovirus virion in the midst of budding from the plasma membrane into the extracellular space. Red arrows represent the positive-strand (i.e., mRNA-sense) viral genomic RNA packaged into each structure to initiate genome replication. Dashed black arrows represent negative-strand RNA in (A) and (B) and negative-strand cDNA in (C), as is initiated prior to virion release for foamy retroviruses.

polymerase into these vesicles, and other points (Ahlquist, 2006). Many, though not all, of these similarities are shared by nodavirus protein A (Dye et al., 2005; Kopek et al., 2007; Miller et al., 2001; Van Wynsberghe and Ahlquist, 2009; Van Wynsberghe et al., 2007).

Particularly striking are the parallels between the above positive-strand RNA virus RNA replication complexes and assembling virions of the foamy retroviruses (Figure 5C). Foamy virus replication is distinct from that of orthoretroviruses such as HIV in several ways (Delelis et al., 2004; Linial, 1999). For example, whereas orthoretroviruses release virions carrying an RNA genome and delay reverse transcription until after entering a newly infected cell, newly assembled foamy retrovirus virions reverse transcribe their encapsidated RNA prior to virion release (Moebes et al., 1997). Thus, foamy retrovirus virions are actively involved in genome replication within the same cell in which they assemble, further paralleling positive-strand RNA virus replication complexes such as the BMV and FHV spherules described above.

Another distinction is that, whereas orthoretoroviruses translate their reverse transcriptase (Pol) as a fusion protein with Gag, foamy viruses translate Gag and Pol as separate proteins from independent mRNAs. This allows separate regulation of Pol expression and encapsidation, similar to some positive-strand RNA viruses such as BMV. Recruitment of foamy virus Pol depends on C-proximal determinants in the Gag protein sequence (Lee and Linial, 2008), similar to the recruitment of BMV 2a^{pol} to RNA replication complexes by the C terminus of BMV 1a (Chen and Ahlquist, 2000; Kao and Ahlquist, 1992).

From an evolutionary perspective, the similarities of positive-strand RNA virus replication complexes with the replicative cores of dsRNA and retrovirus virions suggest that all of these viruses may have diverged from a common precursor that also used a viral protein shell to organize and sequester the replication of an mRNA-sense genomic RNA template. In subsequent evolution, these viruses would then have diverged with regard to which replication cycle intermediate to export in infectious virions: for retroviruses and dsRNA viruses, the RNA replication complex before and after negative-strand synthesis, respectively, and for positive-strand RNA viruses, the mRNA-sense genomic RNA before assembly into the replication complex (Ahlquist, 2006; Schwartz et al., 2002).

Similarities with DNA Virus Replication Factories

Building novel intracellular structures to support viral replication is an integral part of the life cycle of many, if not all, viruses. Unlike the RNA viruses discussed above, most DNA viruses replicate their genomes inside the nucleus. Nevertheless, many of these DNA viruses also assemble cytoplasmic factory-like structures to complete their replication cycles and assemble progeny virus, often in close vicinity to ER membranes where viral proteins are produced (Novoa et al., 2005).

Moreover, unusually among large double-strand DNA viruses, poxviruses such as vaccinia virus carry out their replication entirely in the cytoplasm in membrane-bound viral complexes (Schramm and Locker, 2005). Upon infection, vaccinia virus cores are released and accumulate in close proximity to ER membranes. The incoming genomic DNA leaves the core and preferentially associates with the cytosolic side of the ER membranes (Mallardo et al., 2001). DNA replication is initiated in distinct cytoplasmic sites, often referred to as viral factories, formed through gradual envelopment by rough ER membranes. Occasional small gaps in the surrounding membranes have been observed, presumably allowing the exchange of molecules between the interior DNA replication compartments and the cytoplasm (Tolonen et al., 2001). After completion of this ER wrapping, the viral DNA replication complexes expand in size, perhaps indicative of active DNA replication (Tolonen et al., 2001).

In further analogy with positive-strand RNA and dsRNA viruses, poxvirus genome replication, transcription and translation, and virus assembly are all coordinated within or associated with the DNA replication factories. Although early viral mRNAs are transcribed in the original viral cores (Mallardo et al., 2001), intermediate and late viral mRNAs concentrate in the viral DNA factories and closely associate with ribosomes and translation initiation factors to produce the many different viral proteins (Katsafanas and Moss, 2007). Late in infection, the ER around the viral factories disassembles, coinciding with a dramatic decrease in DNA synthesis and the formation of virion precursors (Tolonen et al., 2001).

Concluding Remarks

The studies reviewed above have substantially enhanced the understanding of the replication structures and pathways of many important viruses and have revealed some common

principles. Simultaneously, many fundamental questions remain or have become evident from this work. Among these unresolved questions are the detailed molecular mechanisms by which specific viruses target their replication factors and their RNAs to particular membranes or other intracellular sites to assemble replication complexes or factories, as well as how different viruses orchestrate the varied and often complex membrane rearrangements associated with their replication processes. Related issues include the specific advantages or adaptations associated with the use by diverse viruses of different intracellular sites for similar replication purposes. Different positive-strand RNA viruses, e.g., variously assemble their RNA replication complexes on distinct secretory, endosomal, or organellar membranes, and they and other viruses show a similar diversity in the sites used for virion assembly and/or budding. However, the implications of such choices for replicative efficiency, virus-host interactions, and pathology remain poorly understood. Recent findings on how picornaviruses, flaviviruses and coronaviruses manipulate components of the secretory pathway and related pathways to create novel membrane environments with specific lipid enrichments and other replication-supportive characteristics are examples of these essential research directions (Belov et al., 2007; Hsu et al., 2010; Reggiori et al., 2010). Such efforts will be critical to identify and understand the roles of cellular factors and molecular pathways in efficient viral replication.

A second class of challenges and opportunities is associated with using growing knowledge in these areas to improve virus control or beneficial uses of viruses. For virus control, growing recognition of the intimate coordination of many successive virus replication steps with each other and with cellular pathways offers many additional points at which to disrupt infection. In this regard, one important area will be further defining the roles and interactions of viral replication compartments as barriers to host defenses, including host systems for detecting viruses through dsRNA, etc., and for impeding virus replication, such as through RNA silencing or certain interferon-stimulated pathways. Though the emerging complexities exceed the expectations of earlier stages of investigation, such questions offer challenging but satisfying directions and a fulfilling future for these important areas in the cell biology of virus replication.

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