Review

Assortment and packaging of the segmented rotavirus genome

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The rotavirus (RV) genome comprises 11 segments of double-stranded RNA (dsRNA) and is contained within a non-enveloped, icosahedral particle. During assembly, a highly coordinated selective packaging mechanism ensures that progeny RV virions contain one of each genome segment. *Cis*-acting signals thought to mediate assortment and packaging are associated with putative panhandle structures formed by base-pairing of the ends of RV plus-strand RNAs (+RNAs). Viral polymerases within assembling core particles convert the 11 distinct +RNAs to dsRNA genome segments. It remains unclear whether RV +RNAs are assorted before or during encapsidation, and the functions of viral proteins during these processes are not resolved. However, as reviewed here, recent insights gained from the study of RV and two other segmented RNA viruses, influenza A virus and bacteriophage Φ6, reveal potential mechanisms of RV assortment and packaging.

Random versus selective genome segment packaging

Viruses that maintain their genomes as separate RNA molecules are faced with a daunting challenge during assembly—how to package a full complement of genome segments. Some RNA viruses use a non-selective packaging mechanism in which segments are randomly encapsidated into virions. This random packaging mechanism creates a large number of particles that lack complete genomes and are thus unable to mediate subsequent infections [1].

Other RNA viruses, particularly those with three or more genome segments, have evolved a more sophisticated packaging mechanism whereby each viral RNA is explicitly recognized. Members of the Reoviridae family are thought to use this gene-specific approach to package their genomes of 9, 10, 11, or 12 segments of double-stranded RNA (dsRNA). The strongest evidence in support of selective rather than random packaging for the Reoviridae comes from the observation that the particle-to-plaque forming unit ratio can be quite low [2,3]. Moreover, a Reoviridae member that contains more than one copy of each gene has never been identified, which suggests a precise equimolar process of assortment [4]. Although the exact mechanism by which the Reoviridae achieve assortment is unknown, these viruses share several features with the eight-segmented, negative-strand RNA viruses of the Orthomyxoviridae family (e.g. influenza A virus) and the three-segmented, dsRNA bacteriophages of the Cystoviridae family (e.g. Φ6) [5,6]. In particular, for all these viruses, *cis*-acting elements in single-stranded viral RNA are thought to determine segment selection, and viral proteins play critical roles in orchestrating the assortment and packaging processes. One of the remaining mysteries for Reoviridae is whether they (i) assort their genome segments prior to packaging, similar to influenza A virus, or (ii) package each segment individually into a preformed particle, similar to Φ6. In this review, we discuss the evidence supporting each of these assortment and packaging models for rotavirus (RV), a Reoviridae family member and significant pediatric gastrointestinal pathogen [7].

RV virion architecture and replication cycle

The mature RV virion is a triple-layered particle that encases 11 dsRNA genome segments (Figure 1) [8–12]. The outermost layer of the virion has T=13 icosahedral symmetry and is composed of the VP7 glycoprotein with several embedded copies of the VP4 spike attachment protein [13,14]. The intermediate layer, which also exhibits T=13 symmetry, is made up of VP6 and surrounds a thin T=1 VP2 core shell [14]. Aqueous channels penetrate the VP6 and VP2 layers, enabling divalent cations and nucleotides to access the particle interior [14]. Viral polymerase complexes (PCs) consisting of a single subunit each of the viral RNA-dependent RNA polymerase (VP1) and RNA capping enzyme (VP3) are attached to the inner surface of the VP2 shell, proximal to most if not all of the 12 fivefold axes [10,14,15]. These enzymes are linked to the core shell through interactions requiring the amino-terminal residues of VP2, which form inwardly protruding fivefold hubs [14–18]. The RV dsRNA genome encodes six structural (VP1–4, VP6 and VP7) and five or six nonstructural (NSP1–5 or –6) proteins predicted to be arranged as tubules that spool around the PCs [14,15].

The primary site of RV infection is the small intestinal villi, where the virus replicates in the cytoplasm of mature enterocytes (Figure 2) [13]. Trypsin-like proteases of the gastrointestinal tract cleave the VP4 spike protein into VP5* and VP8*, an event that primes RV for entry into the cell [12,19,20]. During internalization, the outermost virion layer is lost to yield a VP2–VP6 double-layered particle (DLP). Loss of the outer capsid triggers viral PCs within the DLP to become transcriptionally active and produce numerous copies of capped, non-poly(A) plus-strand RNAs (+RNAs) using the minus strands of dsRNAs as templates [21,22]. Each tethered PC is dedicated to transcription of an individual genome segment, but acts in synchrony with the

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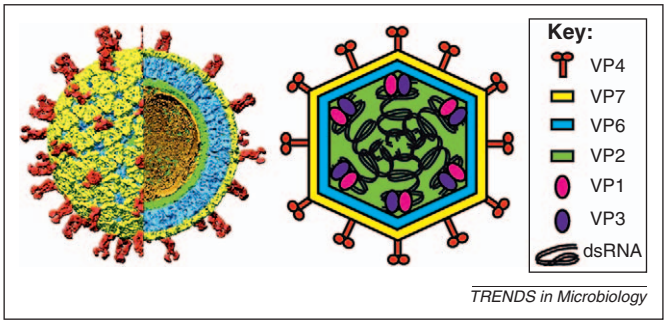

Figure 1. Architecture and protein composition of the RV virion. The left panel shows a cryoelectron micrograph image reconstruction of a mature RV triple-layered particle (TLP) at 9.5 Å resolution and is used with permission from B.V.V. Prasad (Baylor University). A portion of the particle has been computationally removed to reveal the internal virion layers. The smooth external surface is made up of the VP7 glycoprotein (yellow) and is embedded with the VP4 spike attachment protein (red). The intermediate VP6 layer is shown in blue and the thin VP2 core shell is shown in green. Ordered portions of viral dsRNA that line the VP2 shell are shown in gold. Polymerase complex (PC) components, VP1 (the viral polymerase) and VP3 (the viral capping enzyme) are not visualized in this reconstruction, but are predicted to be tethered to the inner surface of VP2 near each fivefold axis. The right panel shows a cartoon schematic of an RV TLP with proteins and dsRNA colored according to the legend.

others to simultaneously create 11 species of +RNA [16,17]. Because the PCs operate independently, transcription is not equimolar; some species of +RNA are produced more abundantly than others [22–25]. RV DLPs are capable of robustly synthesizing transcripts for several hours *in vitro*, which suggests that PCs efficiently re-engage the 3′ ends of the minus-strand templates [26–28]. Newly synthesized RV +RNAs acquire a 5′ cap structure (m^7GpppG) by the activity of the PC component VP3 before their extrusion from the DLP via channels near the fivefold axes [29,30].

Nascent RV +RNAs serve dual roles during the replication cycle, acting as mRNAs for protein synthesis and as templates for genome replication. The intracellular localization of a viral transcript is predicted to largely determine its use in the infected cell [31]. The +RNA products of transcription from incoming DLPs accumulate in the cytosol and are available for translation by host ribosomes into viral proteins. Two viral nonstructural proteins (NSP2 and NSP5) are thought to co-localize around transcribing DLPs, forming dense inclusion bodies termed viroplasms [32,33]. Viroplasm-associated RV +RNAs are selectively packaged into assembled or assembling VP2 cores (detailed below) [31,34]. Following or during encapsidation of the 11 +RNA species, core-associated PCs perform minus-strand synthesis, thereby reconstituting the dsRNA genome inside a pre-virion particle [34]. In contrast to viral transcription, which occurs multiple times on each segment, genome replication is equimolar and produces exactly one of each of the 11 dsRNAs per virion [4]. The timing of genome packaging and replication is regulated in part by interactions between the viral polymerase VP1 and the core shell protein VP2 [16,17]. Engagement of VP1 by VP2 triggers conformational changes in the enzyme that facilitate initiation of RNA synthesis [35–37]. This core shell requirement ensures that the polymerase does not aberrantly replicate the RV genome outside of

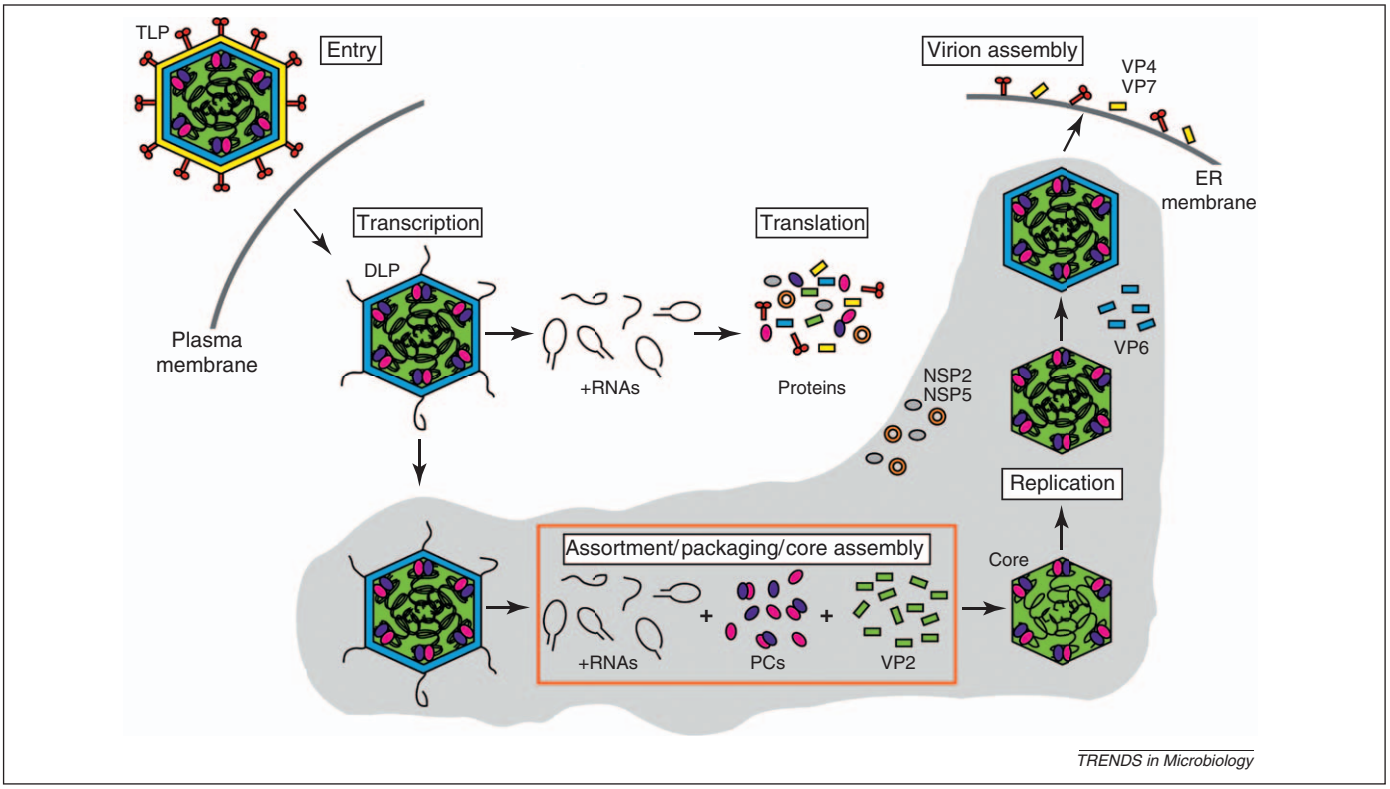

Figure 2. Schematic of the RV replication cycle. During entry of an RV triple-layered particle (TLP) into a host cell, VP4 and VP7 are lost, which results in release of a double-layered particle (DLP). The viral PCs, composed of VP1 (pink spheres) and VP3 (purple spheres), within the DLP interior are transcriptionally active and synthesize multiple copies of 11 species of capped, non-poly(A) +RNAs (black lines). The nascent +RNAs are extruded out of the DLP and deposited into the cytosol, where they serve as templates for translation of viral proteins. Newly made nonstructural proteins NSP2 (orange donut) and NSP5 (gray sphere) form inclusions (viroplasms; gray shaded area) around DLPs, thereby trapping +RNAs that will be used for assortment, packaging and genome replication. Two models are proposed for how the 11 species of +RNA associate with each other, PCs and VP2 (green) to form a fully packaged RV core (red box; Figure 4). During or following their encapsidation, +RNAs are used as templates for replication by the core-associated PCs to recreate 11 dsRNA segments within a pre-virion particle. A VP6 layer (blue) is acquired, and then DLPs bud into the endoplasmic reticulum (ER), during which the outer capsid proteins (VP4 and VP7) are acquired. Mature RV TLPs exit non-polarized cells predominantly by lysis.

particles. Following genome replication, progeny cores acquire a VP6 layer; such DLPs can amplify the replication cycle by supporting secondary rounds of transcription. DLPs eventually bud into the lumen of the endoplasmic reticulum, during which the outer capsid proteins VP4 and VP7 are added [38]. Mature, triple-layered particles are predominantly released from non-polarized cells via lysis, but can be released from polarized cells by both lytic and non-lytic mechanisms [39].

Cis-acting functional elements of RV +RNAs

RV +RNA transcripts contain important sequence and structural elements that promote their (i) recognition by host ribosomes, (ii) assortment and packaging, and (iii) use as templates for genome replication. The 11 RV +RNAs range in size from 0.7 to 3.1 kb and generally contain a single open reading frame (ORF) that is flanked at the 5′ and 3′ ends by untranslated regions (UTRs) (Figure 3) [40]. These molecules contain 5′ cap structures but lack 3′ poly(A) tails and instead end with a highly conserved seven-nucleotide sequence [34]. For group A RV strains, those that cause the majority of disease in humans and animals, the 3′ consensus sequence (3′CS) is UGUGACC. This stretch of nucleotides is an important functional element of viral +RNAs that are located in both the cytosol and the viroplasm. In particular, the 3′CSs of cytosolic +RNAs are bound by NSP3; it has been proposed that this viral nonstructural protein serves as a surrogate of cellular poly(A) binding protein and enhances the translation of RV transcripts in the infected host [41–46]. For viral +RNAs being packaged and used as templates for genome replication in the viroplasm, the 3′CS serves as a critical polymerase recognition element [31,36,37,47]. Binding of the 3′CS by VP1 in the context of a PC presumably facilitates incorporation of +RNAs into core particles [17]. This interaction is also predicted to temporally regulate initiation of minus-strand synthesis [17,37,48]. Specifically, VP1 engages the 3′CS in a manner that holds the terminal end of the +RNA out of register with the enzyme active site, thereby producing a stable, catalytically inactive complex [37]. It has been hypothesized that the auto-inhibited polymerase is subsequently activated when engaged by VP2 during packaging of VP1-bound +RNAs [17,37,48]. Following VP1 activation, the 3′CS functions as a minimal promoter and supports *de novo* initiation of minus-strand synthesis within the confines of a core particle [36,49].

RV +RNAs are predicted to form complex secondary and tertiary structures in infected cells (Figure 3). RV +RNAs used for protein synthesis are probably held in a circular conformation by the interaction of NSP3 (bound to the 3′CS) with the 5′ cap-associated eukaryotic initiation factor (eIF4G) [43,44,46]. Furthermore, computer modeling and RNase mapping experiments suggest that viral +RNAs fold into panhandles via 5′ and 3′ base-pairing [50–52]. In the tertiary +RNA structure, the 3′CS extends from the panhandle as a single-stranded tail [50–52]. Mutations generated in the 3′CS that induce base-pairing with the 5′ end of the +RNA template diminish its replication capacity *in vitro* [50]. Therefore, one function of the 5′–3′ panhandle could be to stabilize the 3′CS in a manner that is sterically accessible to the polymerase. The stable association of the 3′CS tail with VP1 might be further enhanced by recruitment of the 5′ cap of the folded +RNA to a cap-binding site on the polymerase [37].

In addition to roles in genome replication, the predicted panhandle structures of RV +RNAs are likely to contain important functional elements for assortment and packaging. With the exception of the extreme terminal nucleotides,

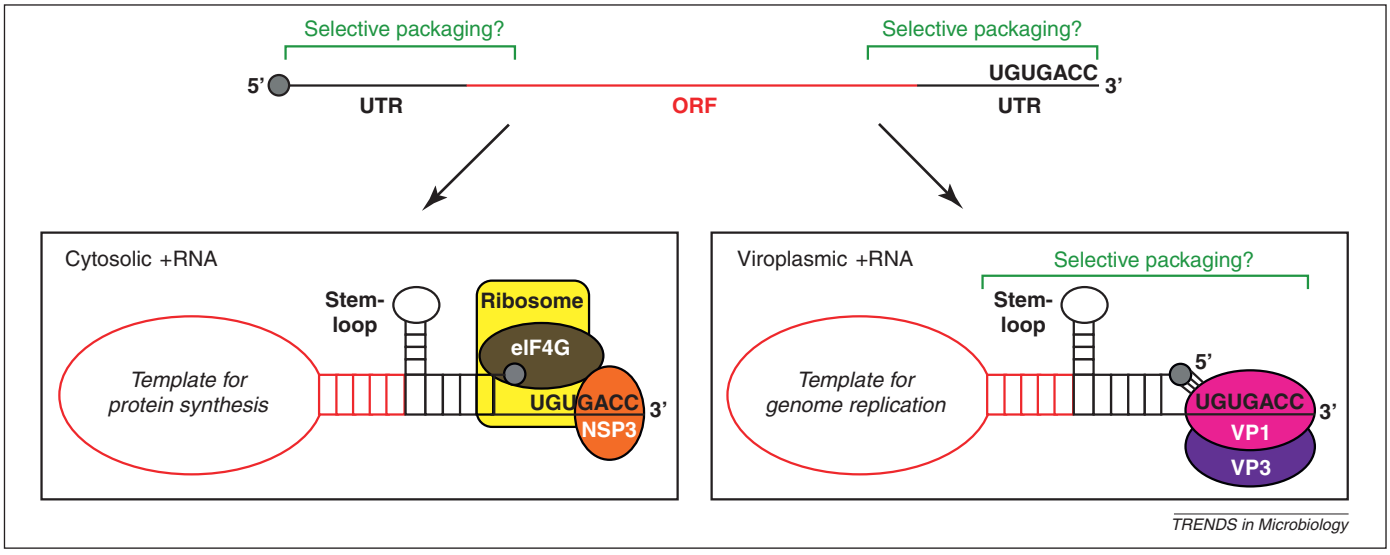

Figure 3. *Cis*-acting elements of RV +RNAs. The top cartoon schematic represents a linear RV +RNA molecule. The central ORF is shown in red, and the 5′ and 3′ UTRs are shown in black. A cap structure (gray) is at the 5′ end of the molecule and the consensus sequence (UGUGACC) is at the 3′ end. Regions of the +RNA thought to be important for selective packaging are indicated using green brackets. The lower left cartoon schematic represents a hypothetical cytosolic +RNA that would be used as a template for protein synthesis. A panhandle structure is formed by base-pairing of the 5′ and 3′ ends, and RNA-specific stem–loop(s) are thought to project from these regions. The 3′ terminus is predicted to be bound by the nonstructural protein NSP3, which itself interacts with eukaryotic initiation factor eIF4G. The NSP3–eIF4G interaction, along with 5′–3′ complementarily, is thought to cause the +RNA to be held in a circular conformation, which might be important for efficient translation by host ribosomes. The lower right cartoon schematic represents a hypothetical viroplasmic +RNA that is selectively packaged into cores and used as a template for genome replication. Similar to the cytoplasmic +RNA, a panhandle structure is formed by base-pairing of the 5′ and 3′ ends, and RNA-specific stem–loop(s) project from these regions. The extreme 3′ end of the template is accessible to the polymerase VP1 (pink sphere) as a single-stranded tail. The 5′ cap of the template is presumed to interact with a cap-binding site on VP1. The VP3 capping enzyme is shown as a purple sphere interacting with VP1. Regions of the folded viroplasmic +RNA thought to be important for selective packaging are indicated using green brackets.

the 11 species of viral +RNA share no homology to each other. Nonetheless, when comparing the same RNA segment of different group A RV strains, the 5′ and 3′ UTRs and neighboring ORF regions show high levels of nucleotide sequence identity. These conserved nucleotides comprise the 5′–3′ panhandles of folded RV +RNAs. Stable stem–loops are predicted within these conserved regions (i.e. within the panhandle) and can be formed by sequences at either the 5′ or 3′ end [50–52]. The observation that the putative stem–loops differ between the 11 +RNAs suggests that they might function as assortment signals. RV variants whose genomes include segments that are missing part or nearly all of the ORF have been identified [53]. The mutant RNAs can still undergo assortment, packaging and replication, which demonstrates that internal ORF nucleotides are not necessary for these processes. Moreover, when passed at a high multiplicity of infection in cell culture, RVs containing genome segments with large duplications tend to appear [53]. This phenomenon indicates that the duplicated +RNAs have advantages over wild-type +RNAs during packaging, possibly owing to repetition of *cis*-acting elements [54]. Still, the functional identification and validation of +RNA elements required for RV assortment await efficient complementary (c)DNA-based reverse genetic and/or *in vitro* packaging systems.

For the closely related Reoviridae family member mammalian orthoreovirus (MRV), an infectious +RNA system has been used to identify important determinants of genome assortment and packaging [55]. In this system, the ORF of an individual MRV +RNA is replaced with that of the reporter chloramphenicol acetyltransferase (CAT). Thus, the *CAT* ORF is flanked by the 5′ and 3′ UTRs of the parental viral +RNA. When transfected into cells expressing the protein encoded by the deleted ORF (along with the nine other MRV +RNAs), the chimeric *CAT* RNA undergoes assortment, packaging and replication. These recombinant MRVs are capable of mediating subsequent infections and expressing the reporter protein in complementing cells. This approach revealed that the 5′ UTR contains the specific packaging signals for at least three different MRV +RNAs (m1, s2, and l1) [56,57]. Like those of RV, conserved 5′ and 3′ terminal sequences of MRV +RNAs are predicted to form 5′–3′ panhandle structures. It is possible that the 5′ packaging signals identified for MRV +RNAs are associated with unique stem–loops that protrude from their panhandles [57]. A similar strategy for analysis of the assortment and packaging signals of +RNAs of the Reoviridae member bluetongue virus indicates that they likewise are located at the ends of the RNA and include sequences of the 5′ and 3′ UTRs and adjoining regions of the ORF [58].

The *cis*-acting RV and MRV +RNA elements important for assortment and packaging might be analogous to those identified for influenza A virus [5]. The eight segments of influenza viral RNA (vRNA) share a common organization, with a long central coding region (in antisense) and relatively short 5′ and 3′ UTRs. The vRNA segments are separately coated by multiple copies of the viral nucleoprotein (NP) and a single copy of the heterotrimeric viral PC to create a ribonucleoprotein (RNP) [59]. Similar to RV +RNAs, the termini of influenza vRNAs are partially base-paired to form panhandle structures, which in turn form corkscrew-like shapes in the context of RNPs [5,59]. RNA-specific packaging signals are predicted to reside in putative stem–loops that are located adjacent to, rather than within, the panhandle of each vRNA and formed by both UTR and terminal ORF nucleotides. The exact manner in which influenza assorts its eight RNPs remains unclear; however, the available data are most consistent with the notion that interactions among the RNA molecules of RNPs mediate this process [60]. It has been proposed that influenza A virus uses a concerted packaging approach, whereby the eight RNPs are first selectively assorted into a genome complex before their incorporation into a budding virion [61].

The dsRNA bacteriophage Φ6 specifically recognizes its three polycistronic +RNAs (s, m, and l) via *pac* sequences located near the 5′ ends [62]. These *pac* sequences are contained within distinctive stem–loop structures and are required for assortment and packaging [6,63–65]. In contrast to influenza and possibly RV +RNAs, the segment-specific *pac* sequences of Φ6 do not overlap with the ORFs, but instead reside entirely with the 5′ UTRs. It is thought that Φ6 +RNAs do not directly interact with each other before encapsidation. Instead, the three +RNAs are each introduced, one by one, into a preformed core particle [64,66]. The reaction is mediated by a hexameric viral motor protein (P4) and is sequential in that the s segment is packaged first, followed by m and then l [64,67,68]. An empty core initially displays a binding site for s, which leads to its recruitment and packaging. Thereafter, a conformational change occurs in the core that reveals a binding site for m. Again, only after packaging of the m segment is the binding site for l uncovered. Once the Φ6 +RNAs are packaged inside the core, internal polymerases convert them into the full-length dsRNA genome segments (S, M, and L) [6]. Forceful expansion of the core shell as a result of the complete packaging of all three +RNA segments triggers Φ6 dsRNA synthesis [65]. Thus, segmented RNA viruses all seem to use *cis*-acting RNA elements as assortment signals, although their overall assortment processes differ markedly.

RV proteins involved in assortment and packaging

The nature of the complexes within which RV +RNAs are assorted and packaged remains poorly understood. Fractionation or immunoprecipitation of RV-infected cells has facilitated physical isolation of core replication intermediates (core RIs) that have the capacity to replicate endogenous +RNAs, recreating the 11-segmented dsRNA genome *in vitro* [69–72]. Importantly, exogenously added RV +RNAs cannot be replicated by the isolated complexes, which suggests that they are captured post-assortment. Core RIs might represent a step in assembly just before the final maturation of a T=1 shell, because the +RNAs are accessible to degradation by single-strand specific RNases [73]. These post-assortment, partially packaged +RNA complexes contain the viral proteins VP1, VP2, VP3, NSP2 and NSP5, any or all of which could play important roles in selective packaging for RV.

VP1, VP2 and VP3 are critical for core assembly and genome replication, and are undoubtedly important for +RNA packaging as well. It has been hypothesized that the VP2 core shell forms by the knitting together of 12

decamer units, which themselves are organized from five asymmetric VP2 dimers [14]. Protruding inward from each fivefold axis are hubs composed of VP2 amino-terminal residues [14]. Deletion of the fivefold hub does not prevent recombinant VP2 from forming core-like particles, but does abrogate VP1 and VP3 encapsidation into those assemblies [18]. Likewise, VP2 lacking amino-terminal residues is not capable of supporting efficient VP1-mediated minus-strand synthesis *in vitro,* which suggests that the fivefold hub plays a role in polymerase activation [35]. Indeed, these biochemical data are consistent with the idea that VP2 fivefold hubs might provide scaffolds upon which PCs function. Still, if VP1 binding of +RNA precedes interactions between the PC and the core shell hub, it is also possible that this VP2 structure plays an indirect role in genome packaging. VP1 is the only known viroplasm-associated protein capable of specifically recognizing RV +RNAs, which supports the notion that this interaction might contribute to selective packaging [36,37,47].

Because NSP2 and NSP5 together induce and maintain viroplasms, they are critical for genome assortment, packaging and replication. However, these nonstructural proteins might also play more direct roles by facilitating or even mediating selective packaging of the 11 RV +RNAs [32]. NSP2 is an octameric protein with strong affinity for single-stranded RNA [74,75]. This protein has helix unwinding and NTPase activities that are not essential for viroplasm formation, but are critical for virus replication [32,76,77]. The helix unwinding activity of NSP2 might help to organize +RNAs for packaging and replication by removing interfering secondary structures. The NTPase activity of NSP2 suggests that this protein could function as a molecular motor by facilitating the insertion of +RNAs into assembling cores in a manner similar to the P4 protein of Φ6 [74]. Compared to NSP2, less is known about NSP5 and its putative role in selective packaging. NSP5 is a dimeric serine/threonine-rich protein that, owing to varying degrees of phosphorylation, exists as multiple isoforms in infected cells [78]. The functional significance of NSP5 phosphorylation remains speculative, because it is unconnected to viroplasm formation [79]. Like NSP2, NSP5 interacts with RNA in a nonspecific manner [80]. These proteins also bind to each other; NSP5 competes with RNA for binding to the tetramer–tetramer grooves on the NSP2 octamer [81]. In addition, biochemical evidence suggests that NSP2 and NSP5 bind VP1 and VP2 [82,83]. Such interactions are likely to aid in the recruitment or retention of core proteins in the viroplasm, and could also help to orchestrate the sequence of events required to assemble progeny cores. For example, during infection, the self-assembly tendency of VP2 must be suppressed, possibly via NSP2/NSP5 interactions, until appropriate packaging and replication events have occurred [84–86].

Models of RV +RNA assortment and packaging

We propose two models of selective +RNA packaging by RV, concerted and core-filling, based on the strategies used by influenza A virus and Φ6, respectively (Figure 4). In the

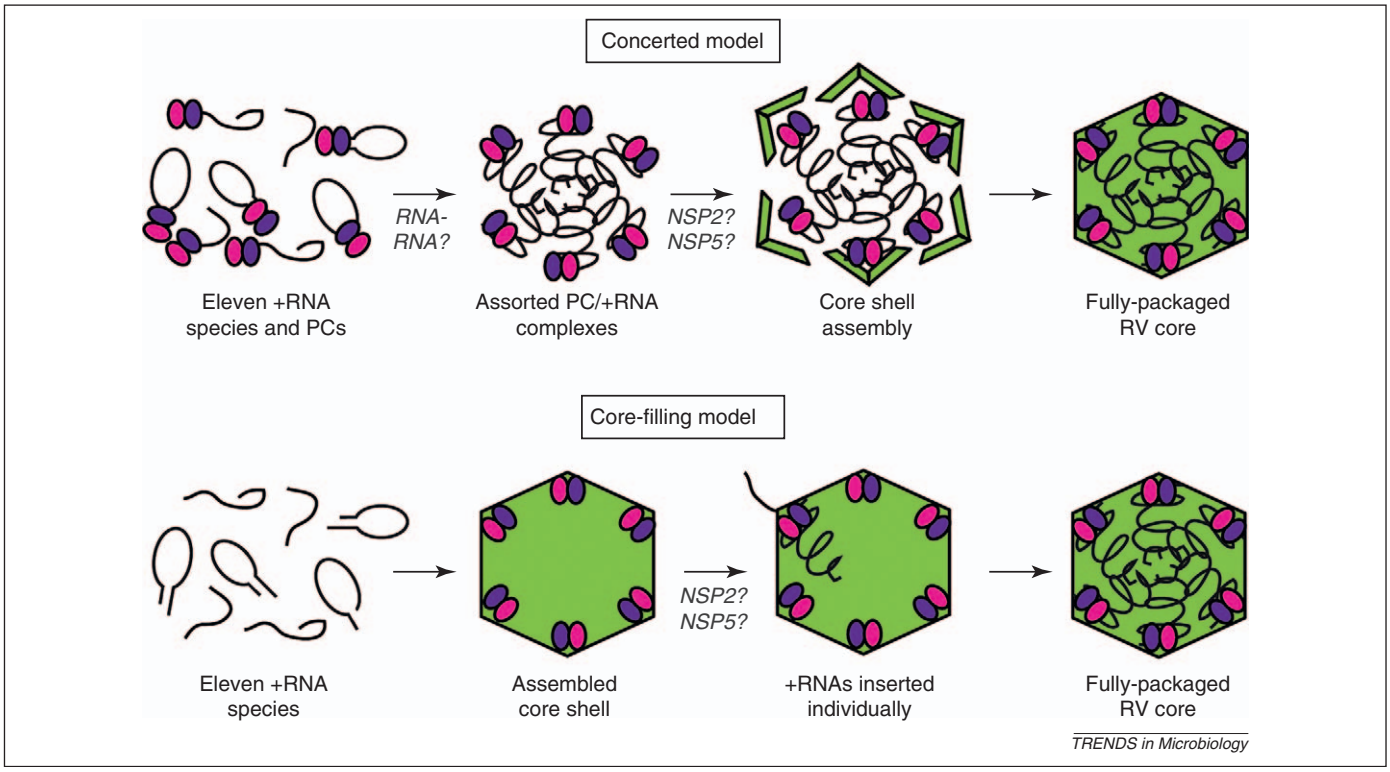

Figure 4. Models of RV +RNA assortment and packaging. Two models of selective +RNA packaging, concerted and core-filling, are proposed for RV based on the strategies used by influenza A virus and Φ6, respectively. In the concerted packaging model (top), 11 species of RV +RNAs (black lines) are bound by PC components VP1 (pink spheres) and VP3 (purple spheres). These PC/+RNAs undergo assortment via gene-specific interactions among the RNA molecules. A VP2 shell (green) then assembles around the assorted PC/+RNAs to create a fully packaged and replication-competent core. NSP2 and NSP5 might function to regulate the timing of core assembly. In the core-filling model (bottom), a VP2 shell (green) containing internally tethered PCs (pink and purple spheres), but lacking nucleic acid, first assembles. Each of the 11 +RNAs (black lines) is then individually inserted into the core, possibly by the functions of NSP2 and/or NSP5. Complete packaging triggers core expansion and initiation of genome replication. The cartoons are meant to illustrate the order of events and not the nature of protein and RNA interactions.

concerted packaging model, assortment is mediated by RNA–RNA interactions and happens before genome packaging. For influenza, vRNAs in the context of RNPs undergo assortment before they acquire an envelope by budding from the plasma membrane. Interactions among the eight vRNA molecules via *cis*-acting stem–loop structures mediate RNA specificity. In the concerted packaging model for RV, each +RNA species made by DLPs within the viroplasm is first bound by a single PC as a result of the affinity of VP1 for the 5′ cap and 3′CS. Aided by interactions with NSP2 and NSP5, the 11 unique PC/+RNAs then undergo assortment in the absence of VP2. In support of this model, complexes have been isolated from RV-infected cells that contain VP1, VP3, NSP2, NSP5 and single-stranded RNA molecules [69,71]. The existence of such pre-core RIs and their capacity to be chased into more complex viral structures suggest that +RNA recognition by PCs precedes VP2 association [69]. Furthermore, the affinity of VP1 for RV +RNA and the manner in which this interaction creates a catalytically inactive complex also support the concerted packaging model [37]. It is possible that the auto-inhibited binding of +RNAs by VP1 allows time for the 11 replication templates to find each other before core shell encapsidation. RV RNA specificity in the concerted packaging model, similar to that for influenza, is mediated by RNA–RNA interactions involving stem–loop structures within the 5′–3′ terminal panhandle of the +RNA molecules. Following assortment, the VP2 core shell assembles around the PC/+RNAs, in turn creating a loosely packaged core RI [69,71,73]. A precise, but as yet unidentified, interaction occurs between VP1 and VP2 that leads to enzymatic activation of the polymerase [35]. Minus-strand synthesis creates the 11 dsRNA genome segments inside a pre-virion particle. However, it is not known whether core RIs become fully mature *T*=1 cores before or during genome replication. *In vitro* studies have shown that core RIs decrease in size and become RNase-resistant as genome replication proceeds [73]. These data suggest that the condensation of VP2 into a tightly closed *T*=1 core shell occurs during minus-strand synthesis. Portions of the +RNA template might extend from the outer surface of the assembling VP2 core and be drawn into the particle as they are replicated.

In contrast to concerted packaging, the core-filling model dictates that the 11 +RNAs undergo ordered insertion into a pre-formed protein shell, similar to the strategy used by some bacteriophages. For Φ6, core particles containing viral polymerases are able to self-assemble *in vitro*. When incubated in the presence of the packaging motor protein P4, Φ6 +RNAs are introduced into the core through a portal located at a specific fivefold axis. Following the introduction of each of the three +RNAs in a sequential manner, expansion of the viral capsid takes place; this conformational change in the core shell induces activation of the internal polymerases. Φ6 minus-strand synthesis occurs inside a particle that is the morphogenic precursor to a mature virion. In the core-filling model for RV, intact but empty *T*=1 core shells with internally tethered PCs assemble *de novo* in viroplasms. Through the NTPase activity of NSP2, each of the 11 +RNAs is individually translocated into the core shells. In this manner, RNA selection is mediated not by interactions among the +RNAs, but by the specific binding of viral protein(s). For example, packaging of one +RNA could expose a binding site for the specific packaging of the next +RNA. Following introduction of the 11 +RNAs, core expansion possibly triggers the PCs to initiate minus-strand synthesis to generate the dsRNA genome.

There are several problems in suggesting a core-filling packaging model for RV. For instance, although empty virus particles can be recovered from infected cells [25], no data have been reported indicating that such particles are precursors of infectious virions, nor have particles been recovered that contain partially packaged or replicated genomes. Moreover, although empty core-like particles can be formed by co-expression of VP1, VP2 and VP3, there are no experimental data suggesting that such particles can be packaged by +RNA *in vitro*, even in the presence of NSP2 [18,84]. In addition, RNA interference experiments have revealed that knockdown of VP1 expression in infected cells leads to accumulation of empty particles [25], a result that is not consistent with the core-filling model, in which VP1 has no anticipated role in +RNA translocation into the core. It also seems unreasonable to propose that the NSP2 octamer is the equivalent of the Φ6 P4 packaging motor. In particular, whereas +RNA moves into the Φ6 core through a central hole located in the P4 hexamer [67,68], the RNA-binding sites are located on the surface of the NSP2 octamer, which suggests that NSP2 is not a functional homolog of P4 [81]. Finally, in this packaging model, it is difficult to imagine how efficient segment recognition could be achieved by the sequential exposure of 11 separate +RNA interaction sites on the core. Notably, +RNAs of partially packaged RV cores (i.e. core RIs) are RNase-sensitive, but those of partially packaged Φ6 cores are RNase-resistant [86]. This biochemical observation suggests that even if RV uses a core-filling approach, significant differences exist when compared with Φ6.

Concluding remarks and future directions

One of the most interesting topics of RV biology is the mechanism by which the virus assorts, packages and replicates its segmented genome. The evidence to date is most consistent with a concerted packaging model, whereby the 11 +RNAs interact with each other using *cis*-acting sequence and structural elements before encapsidation within a core. Regardless of the mechanism, packaging of a segmented genome complicates the assembly process for RV relative to other viruses whose genomes consist of a single piece of nucleic acid. Even so, genome segmentation confers distinct evolutionary advantages, because it affords the opportunity for reassortment during co-infection. In particular, segment exchanges could allow RVs to acquire advantageous genes and thus to rapidly adapt to selective pressures. Yet, reassortment between divergent strains requires that critical protein–protein interactions be maintained

Box 1. Restrictions on RV segment reassortment

The capacity of RVs to reassort their genome segments during co-infection is predicted to be a major driving force in the evolution of this pathogen. Yet, there exist certain RV strain combinations that seem to be incapable of reassortment under experimental conditions and in nature. In particular, there has been no demonstration of gene reassortment between RV strains belonging to different serogroups (A–G). Although the reason for the observed restriction is not known, it is possible that multiple determinants, both direct and indirect, prevent intergroup genetic exchange. Direct determinants refer to the capacity of 'foreign' RV +RNA to be packaged and replicated by proteins from another group. For example, co-infection of a host cell with group A and C RVs must provide an opportunity for physical mixing of their gene segments in common viroplasms, stable interaction of their +RNA and core proteins during assortment and packaging, and an ability of their polymerases to replicate each other's template RNAs. Indirect determinants of gene reassortment restriction relate to the capacity of the exchanged genes to function together in the new strain. Emerging evidence suggests that whereas group A RVs readily undergo reassortment events, there are selection pressures for the maintenance of certain sets of genes (i.e. preferred genome constellations) [87,88]. One of these pressures relates to how well the proteins encoded by the new RV interact during subsequent rounds of replication. For group A RV reassortants, the pressures are subtle and might be seen during the evolution of circulating viruses. By contrast, because RV groups encode very divergent proteins, intergroup reassortants that break preferred constellations might not be capable of carrying on a productive infection [89]. Given that gene reassortment has the potential to create novel and possibly more pathogenic RV strains, future studies in this area are warranted.

during viral replication (Box 1). Recent large-scale genomics studies suggest that co-circulating human RVs do not freely reassort genome segments, but instead have preferred RNA sets (i.e. genome constellations) [87]. It is possible that the functional constraints imposed by viral protein and RNA interactions during replication represent important determinants of viral fitness, thereby affecting whether or not a reassortant will occur in the human population [88,89]. Future research is necessary, not only to define the RNA and protein interactions critical for RV assortment and packaging, but also to elucidate the limitations on segment exchange between genetically divergent strains (Box 2). Efficient single-gene reverse genetic methods that are now being developed for RVs will undoubtedly provide a scientific platform for such studies.

Box 2. Questions for future research

- Do RVs use a concerted or core-filling approach to assortment and packaging?
- Where are the packaging signals located in RV +RNAs?
- What are the functions of viral proteins (VP1, VP2, VP3, NSP2 and NSP5) during the assortment, packaging and replication of the 11-segmented RV genome?
- Do RNA–RNA or protein–RNA interactions drive RV +RNA assortment?
- What are the molecular determinants of RV gene reassortment restriction?
- How does gene reassortment contribute to RV diversity and evolution?

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