

Parallels among positive-strand RNA viruses, reverse-transcribing viruses and double-stranded RNA viruses

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Abstract | Viruses are divided into seven classes on the basis of differing strategies for storing and replicating their genomes through RNA and/or DNA intermediates. Despite major differences among these classes, recent results reveal that the non-virion, intracellular RNA-replication complexes of some positive-strand RNA viruses share parallels with the structure, assembly and function of the replicative cores of extracellular virions of reverse-transcribing viruses and double-stranded RNA viruses. Therefore, at least four of seven principal virus classes share several underlying features in genome replication and might have emerged from common ancestors. This has implications for virus function, evolution and control.

Positive-strand RNA virus

A virus, the infectious virions of which contain the genome in a single-stranded, messenger-sense RNA form.

Despite continuing advances, established and emerging viruses remain major causes of human disease, with dramatic costs in mortality, morbidity and economic terms. In addition to acute diseases, viruses cause at least 15–20% of human cancers^{1,2} and are implicated in neurological and other chronic disorders. One of many challenges in controlling viruses and virus-mediated diseases is that viruses show an amazing diversity in basic characteristics and life cycles, including differences in virion structure, replication strategies, genetic organization, gene expression and many other fundamental processes. Therefore, even the very processes against which antivirals are targeted often differ radically among virus classes. Inherent in this remarkable variety are intriguing issues about the multiplicity of virus origins and the functional and evolutionary relations of existing viruses. Such issues have practical as well as academic importance, as underlying similarities among virus classes might serve as a foundation for broader-spectrum antiviral strategies.

One of the most elemental differences among viruses is their diversity in genome replication and encapsidation strategies, which define seven major classes (FIG. 1). Some viruses replicate their genomes solely through DNA intermediates, packaging these genomes in infectious virions either as double-stranded (ds)DNA or single-stranded (ss)DNA. By contrast, most viruses replicate their genomes solely through RNA intermediates. Such RNA viruses are divided into three classes based on whether their virions package the genome as mRNA-sense (positive-strand) ssRNA, antisense (negative-strand)

ssRNA or dsRNA. Other viruses replicate by interconverting their genomes between RNA and DNA. The virions of such reverse-transcribing viruses always initially package the RNA forms of their genomes, and either might (for example, hepadnaviruses and foamy retroviruses) or might not (for example, orthoretroviruses) reverse-transcribe the RNA into DNA before the virion exits the initially infected producer cell.

Viruses in each of these seven classes tend to share additional features, such as gene-expression strategies and so on, that further cluster and differentiate their members from the other classes, showing that these classes represent meaningful, functionally distinct groupings and probable evolutionary lineages. Some of these variations arise because the type of nucleic acid delivered by the virion to a target cell dictates early infection and gene-expression steps. For example, to initiate viral gene expression, dsRNA virus virions and negative-strand RNA ((-)RNA) virus virions contain viral polymerases that transcribe the genome into translatable mRNA, and reverse-transcribing-virus virions contain polymerases that copy the genome into cell-transcribable DNA (BOX 1). Positive-strand RNA ((+)RNA) viruses, the virions of which deliver immediately translatable messenger-sense RNAs, encapsidate their RNA without a polymerase and form strictly intracellular RNA-replication and mRNA transcription complexes (BOX 1).

Despite these and other differences, recent results have revealed fundamental parallels in the genome-replication processes of certain (+)RNA viruses, dsRNA viruses and reverse-transcribing viruses. In particular, the

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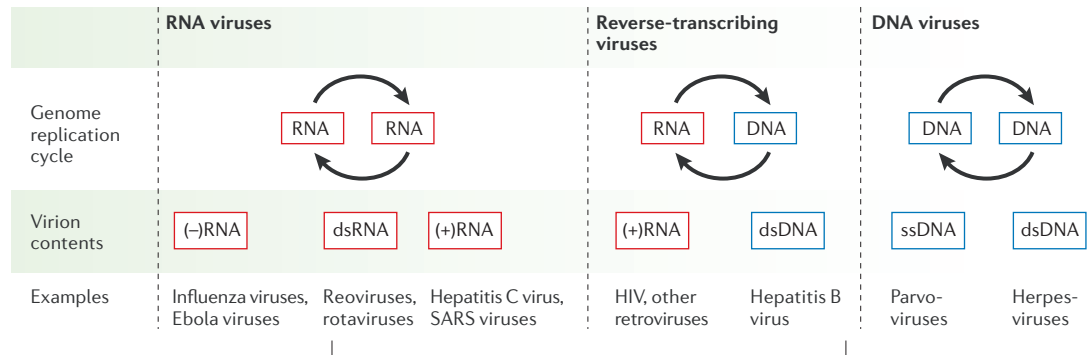


Figure 1 | **Seven classes of virus distinguished by genome replication and encapsidation strategies.** The bracket highlights the four virus classes emphasized in this review. (+)RNA, positive-strand RNA, which is single-stranded RNA of the same polarity as viral mRNA; (-)RNA, negative-strand RNA, which is single-stranded RNA of anti-mRNA polarity; dsRNA, double-stranded RNA; SARS, severe acute respiratory syndrome.

intracellular RNA-replication complexes of some, if not many, (+)RNA viruses share several similarities with the replicative cores of virions from both dsRNA viruses and reverse-transcribing viruses. This review outlines these similarities and their potential implications for virus function and evolution. As primary examples, we review similarities among (+)RNA viruses in the alphavirus-like superfamily, the dsRNA reoviruses and the retroviruses. Other shared characteristics with similar evolutionary implications have been recognized recently among certain other (+)RNA viruses, the dsRNA birnaviruses and the reverse-transcribing hepadnaviruses, including parallels in viral RNA-polymerase structure, capsid proteins and protein priming of genome replication³⁻⁷.

Negative-strand RNA ((-)RNA) viruses (FIG. 1) also share similarities with some of the basic features reviewed here, suggesting that the functional and evolutionary parallels discussed below might be extended further. These possibilities are not discussed here in detail for reasons of space. In addition, whereas (+)RNA viruses, dsRNA viruses and reverse-transcribing viruses each use identical (+)RNA molecules as genome-replication intermediates and mRNAs, (-)RNA viruses are distinguished by using different forms of (+)RNA for these functions.

(+)RNA virus and retrovirus parallels

tRNAs and genome replication. One of the first similarities recognized between the replication of retroviruses and (+)RNA viruses was the role of tRNAs in initiating retroviral reverse transcription and of tRNA-like elements in initiating RNA replication by a subset of (+)RNA viruses such as the bromoviruses^{8,9} (FIG. 2). Bromoviruses, discussed further below, have three genomic RNAs with highly conserved, structured, tRNA-like 3' ends (FIG. 2b). These 3' ends terminate in 3'-CCA_{OH} sequences that are completed by tRNA-nucleotidyl transferase, they are specifically aminoacylated *in vitro* and *in vivo* with tyrosine, and they contain the promoter for (-)RNA synthesis¹⁰⁻¹³.

tRNA-related sequences initiate negative-strand synthesis for genomic RNA replication in both retroviruses and (+)RNA viruses, but the mechanisms are distinct. For

retroviruses, a cellular tRNA covalently primes negative-strand cDNA synthesis, whereas for the relevant (+)RNA viruses, a viral tRNA-like element serves as a recognition site and template for (-)RNA synthesis that is initiated *de novo*, without a primer. A natural intermediate and potential evolutionary link between these processes was identified by Lambowitz and colleagues, who showed that a *Neurospora crassa* mitochondrial retroplasmid initiates reverse transcription without a primer at the tRNA-like 3' end of its genomic RNA, paralleling negative-strand initiation by (+)RNA viruses¹⁴.

Membrane-associated RNA-replication complexes. As noted above, (+)RNA viruses differ from retroviruses and other RNA viruses in that they do not encapsidate their polymerases in extracellular virions. Nevertheless, emerging results show that similarities between (+)RNA-virus RNA replication and retrovirus reverse transcription are not limited to aspects of negative-strand initiation and the general similarities of RNA and DNA polymerases. Instead, as detailed below, (+)RNA-virus RNA replication occurs in virus-induced compartments which have many similarities to the replicative cores or capsids of retrovirus virions.

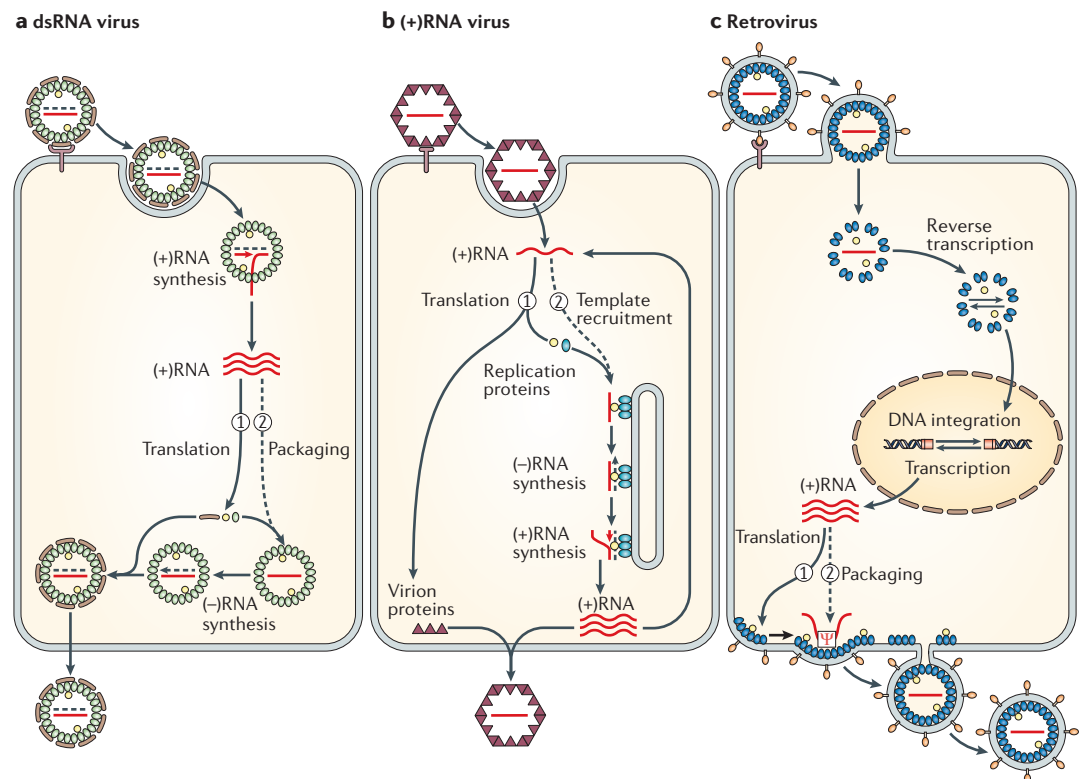
(+)RNA-virus replication is invariably localized to intracellular membranes. Different (+)RNA viruses target distinct but usually specific membranes, such as those of the endoplasmic reticulum (ER)¹⁵⁻¹⁹, endosomes^{20,21}, mitochondria²² or chloroplasts²³. RNA replication is usually associated with rearrangements of these target membranes, often giving rise to membrane invaginations, single- or double-membrane vesicles, membrane-bound vesicle packets and other structures.

For many (+)RNA viruses, RNA synthesis localizes to membranes bearing 50-70-nm vesicular compartments that are invaginated away from the cytoplasm into the lumen of the affected secretory compartment or organelle. Such invaginations, termed spherules, were first visualized in early electron microscopy (EM) studies of alphavirus-infected cells^{20,21}. There are many other examples of such invaginations in and beyond the alphavirus-like superfamily, such as those associated with bromoviruses^{24,25}, nodaviruses²² and tymoviruses²³.

Negative-strand RNA virus
A virus, the infectious virions of which contain the genome in a single-stranded, anti-messenger-sense RNA form.

Retroplasmid
A DNA plasmid that replicates by transcription and reverse transcription of an RNA intermediate.

Box 1 | Distinct life cycles of dsRNA viruses, (+)RNA viruses and retroviruses



All three classes of virus replicate through positive-strand RNA ((+)RNA) intermediates (red strands in the figure) that are templates for both translation and genome replication. For each class, the figure shows a simplified, representative life cycle.

Double-stranded (ds)RNA viruses

As shown in part **a** of the figure, virus attachment and endocytosis deliver a virion core that contains viral genomic dsRNA and viral RNA polymerase (yellow) into the cytoplasm. The core transcribes and extrudes (+)mRNAs that are first translated (1) and then packaged (2) by the resulting viral proteins into new virion cores. Cores mature by synthesizing negative-strand (-)RNA (dotted strand) and adding exterior proteins. They exit by cell lysis or secretion.

(+)RNA viruses

As shown in part **b** of the figure, endocytosed virions release messenger-sense genomic RNA into the cytoplasm for translation. Newly translated viral RNA-replication proteins recruit this genomic RNA into a membrane-associated, intracellular RNA-replication complex. Small amounts of (-)RNA are produced and used as templates to greatly amplify viral (+)RNA, which is encapsidated into new progeny virions.

Retroviruses

As shown in part **c** of the figure, virion attachment and envelope fusion release a subviral complex that contains viral genomic (+)RNA and reverse transcriptase (yellow). After cDNA synthesis by reverse transcription, proviral cDNA is integrated into the host chromosome and transcribed to produce (+)RNA that is translated (1) and then packaged (2) into new virions that are released by budding.

As illustrated in FIG. 3a–c, for nodaviruses and bromoviruses, such spherules frequently occur in contiguous clusters and are often light-bulb-shaped structures, the interiors of which are connected to the cytoplasm through membranous necks.

Parallels with retrovirus capsids. Retroviruses package their reverse transcriptase (also designated Pol) and their genomic RNA templates into membrane-enveloped capsid shells assembled by the major capsid protein, Gag^{26–28} (FIG. 4a). For most retroviruses, these capsids bud from the cell together with viral envelope proteins and are delivered to new cells by infection, giving rise to

intracellular complexes in which reverse transcription occurs^{29–31}. For foamy retroviruses and retrovirus-like LTR (long terminal repeat) retrotransposons, reverse transcription occurs in such capsids without leaving the producer cell^{32–34}. Such capsids contain hundreds³⁵ to thousands³⁶ of Gag proteins and approximately 10–20-fold fewer Gag–Pol fusion proteins^{28,37}. Retrovirus genomic RNAs are selectively packaged in these capsids by Gag interaction with specific *cis*-acting signals, often designated as Ψ ^{38,39}.

Recently, links between the intracellular spherules of (+)RNA viruses and retrovirus capsids emerged from studies of brome mosaic virus (BMV) RNA replication.

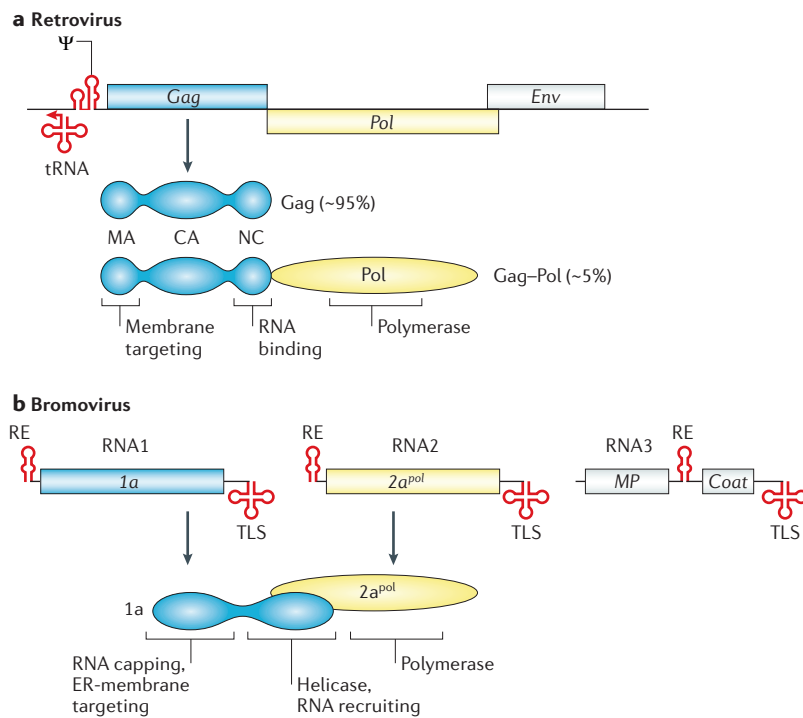


Figure 2 | Schematic overview of retrovirus and bromovirus genomes. a | Schematic of the genomic RNA of a simple retrovirus and encoded virion proteins Gag and Gag–Pol. **b** | Schematic of bromovirus genomic RNAs 1, 2 and 3 and encoded RNA-replication factors 1a and 2a^{pol}. CA, capsid; Env, envelope-protein gene; ER, endoplasmic reticulum; MA, matrix; NC, nucleocapsid; RE, RNA template recruiting element for genomic RNA replication; TLS, 3' tRNA-like sequence, which contains the promoter for negative-strand RNA synthesis; tRNA, host tRNA primer for negative-strand cDNA synthesis; Ψ, RNA-packaging signal.

BMV is the type member of the bromoviruses and a representative member of the alphavirus-like superfamily of (+)RNA viruses. This superfamily includes many viruses that infect animals or plants, all of which share three conserved protein domains that are involved in RNA replication^{40,41}. In BMV, these domains are distributed across two replication proteins, **1a** and **2a^{pol}** (FIG. 2b), that co-localize on ER membranes at the sites of viral RNA synthesis^{15,16}. 1a contains an RNA-helicase-like domain and an RNA-capping domain with m⁷G methyltransferase and covalent m⁷G binding activities required for capping of viral RNAs *in vivo*^{42–44}. 2a^{pol} contains a central polymerase domain.

Protein 1a is a multifunctional protein with central roles in the genesis and operation of BMV RNA-replication complexes (FIG. 4b). In the absence of other viral factors, 1a localizes to ER membranes¹⁶ and induces invagination of the spherular replication compartments⁴⁵. 1a also recruits nascent or full-length 2a^{pol} to ER membranes by an interaction between the 1a helicase-like domain and the N-terminal extension that precedes the 2a^{pol} polymerase domain^{46–48}.

Protein 1a also transfers BMV genomic RNA-replication templates to a new state, which was first recognized because the stability and accumulation of BMV genomic RNAs was dramatically increased by 1a co-expression in yeast^{49–51}. Subsequent work showed that 1a induces the transfer of BMV genomic RNAs to

a novel, membrane-associated, nuclease-resistant state⁴⁵. The location of the nuclease-resistant RNA and the site of RNA synthesis seem to be the spherule interior, as (+)RNA and (–)RNA templates and nascent BMV RNAs show identical membrane association and nuclease resistance, and immunogold EM localizes 5-bromo-UTP (BrUTP)-labelled nascent RNAs to the spherule interior⁴⁵.

Recruitment of BMV genomic RNAs to the RNA-replication complex by 1a is controlled in *cis* by internal (RNA3) or 5' proximal (RNA1 and RNA2) recruitment elements (REs) on each genomic RNA (FIG. 2b), which are necessary and sufficient for 1a responsiveness^{51,52}. Mutational studies show that the RE activity of BMV RNA derivatives in 1a-induced recruitment is closely linked to their relative template activity in (–)RNA synthesis and full RNA replication^{51,52}. Therefore, 1a-responsive RNA-template recruitment seems to be a crucial step that precedes replication.

As shown in FIG. 4a,b, the roles of 1a, 2a^{pol} and the *cis*-acting REs parallel the roles of Gag, Pol and Ψ in retrovirus replication. Similar to Gag, 1a localizes to the cytoplasmic face of membranes as a peripheral membrane protein⁵³, self-interacts⁵⁴, and is the sole viral factor required to induce membrane invagination⁴⁵. Immunogold EM labelling and biochemical analyses show that each spherule contains hundreds of 1a proteins⁴⁵. The resulting spherules (FIG. 3c) are remarkably similar to the necked vesicles that result when retrovirus budding is arrested by mutations in the Gag late domain that recruits host factors required for membrane breakage and fusion⁵⁵. Moreover, the high multiplicity of 1a in spherules, its strong membrane association and self-interaction, and the dependence of endocytic and secretory vesicle formation and enveloped-virion budding on protein coats or shells^{56,57} indicate that 1a might induce membrane invagination by forming a capsid-like shell similar to that of Gag. Similarly, the 1a–RE interaction in RNA-template recruitment parallels the Gag–Ψ interaction in retrovirus RNA packaging. Like retrovirus Pol, BMV 2a^{pol} is not required for spherule formation or RNA recruitment. However, when expressed, 2a^{pol} is incorporated into the replication complex in a 1a/2a^{pol} ratio of ~25, similar to the Gag/Pol ratio of 10/20 (REFS 37,45).

Similarities with other (+)RNA viruses

The universal association of (+)RNA-virus RNA replication with modified intracellular membranes, often in association with membrane invaginations, and other shared features imply that the RNA-replication complexes of a wide variety of (+)RNA viruses might use principles that are similar to those illustrated in FIG. 4b. Such parallels include the following: both alphavirus- and cucumovirus-induced membrane spherules contain dsRNA^{25,58}; BrUTP labelling implies that the interiors of alphavirus spherules are the sites of viral RNA synthesis⁵⁹; hepatitis C virus and coronavirus (–)RNA-replication templates are in a membrane-associated, nuclease-resistant state^{60–62}; hepatitis C virus replication proteins are present in active replication complexes at >100-fold excess over viral RNA and are sequestered in a protease-resistant

BrUTP labelling

Labelling RNA by replacing the substrate UTP with 5-bromo-UTP (BrUTP). The labelled RNA can be localized using electron microscopy following immunogold labelling with an antibody directed against BrU.

state^{61,62}; **poliovirus** polymerase and possibly other replication factors self-oligomerize in extended lattices⁶³; and tombusvirus genomic RNA possesses an internal *cis* signal for initial template recruitment and separate 3' terminal sequences for negative-strand initiation⁶⁴.

Furthermore, just as for retrovirus Gag and BMV 1a, the membrane structures associated with genome replication by many (+)RNA viruses can be induced by a subset of viral RNA-replication proteins that do not include the polymerase. Examples include the RNA-replication-associated membrane structures induced by alphavirus nsP123 (REF. 65), arterivirus nsp2 and nsp3 (REF. 66), hepatitis C virus nsP4B (REFS 19,67) and picornavirus 2BC (REFS 18,68,69).

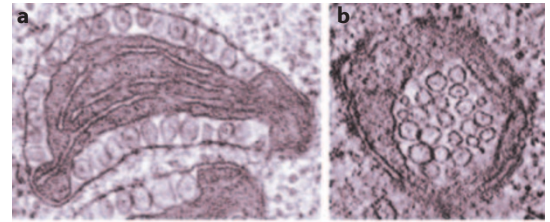
As explained further below, the parallels among (+)RNA-virus RNA-replication complexes, retrovirus capsids and dsRNA-virus capsids (FIG. 4) imply possible mechanistic explanations for several common features of (+)RNA-virus RNA replication, such as the ability of the replication complex to retain (–)RNA templates for positive-strand synthesis, differential regulation of (–)RNA and (+)RNA synthesis, and frequent down-regulation of polymerase expression.

Pol regulation in retroviruses and (+)RNA viruses. In retrovirus genomes, the Pol open reading frame (ORF) follows that of Gag and is expressed by a rare translational frameshift or translational readthrough event, generating an ~20-fold ratio of Gag/Gag–Pol (FIG. 2a) that is incorporated into the final virion (FIG. 4a). This highly asymmetric ratio regulates the free volume and other parameters of the capsid, and is functionally important, as increasing the level of Gag–Pol relative to Gag inhibits retrovirus virion assembly, release and infectivity^{37,70,71}.

Similarly, many (+)RNA viruses downregulate expression of their polymerase relative to RNA-replication proteins related in sequence and/or functions to 1a. The finding that BMV 1a parallels Gag in acting at high multiplicity to induce the RNA-replication compartment (FIG. 4a,b) implies that such polymerase downregulation might satisfy functional requirements similar to those governing the Gag/Gag–Pol ratio in retrovirus capsids. Like retroviruses, many (+)RNA viruses use translational readthrough or frameshift to downregulate polymerase expression. Well-studied examples include the animal alphaviruses and arteriviruses and plant tobamoviruses and tombusviruses. In each case, the ORF upstream to that of polymerase encodes a protein(s) with parallels to 1a. Specifically, alphavirus nsP123 and tobamovirus p130 are homologous to 1a (REF. 40), and nsP123 is sufficient to induce membrane spherules⁶⁵; arterivirus ORF1a proteins induce membrane rearrangements associated with RNA replication⁶⁶; and tombusvirus p33 self-interacts and directs membrane association of itself, viral RNA and polymerase⁷². As with Gag and Pol, increasing expression of the polymerase-containing fusion proteins at the expense of the upstream membrane-interacting proteins inhibits tobamovirus and alphavirus replication^{73,74}.

Unlike the above examples, bromoviruses express 1a and 2a^{pol} from separate genomic RNAs (FIG. 2b). In this

Nodavirus-modified mitochondria



Bromovirus-modified nuclear membranes

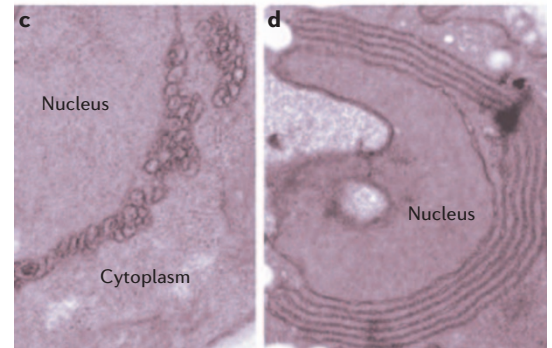


Figure 3 | Electron micrographs of membrane rearrangements associated with nodavirus and bromovirus RNA replication. **a** | Mitochondrion in a flock-house-nodavirus-infected *Drosophila* cell, showing the typical 50–70-nm, light-bulb-shaped spherular invaginations of the outer mitochondrial membrane into the expanded lumen between the inner and outer membranes. Reproduced with permission from REF. 22 © (2001) American Society for Microbiology. **b** | Mitochondrion in a flock-house-nodavirus-infected *Drosophila* cell that has been sectioned perpendicular to the axis of the spherule necks, rather than parallel to these axes as in panel **a**. This view reveals a ‘vesicle packet’ appearance (B. Koepke and P.A., unpublished results). Note that invagination into the lumen of any closed membrane compartment such as the endoplasmic reticulum (ER) or mitochondrial envelope creates a spherule interior that remains connected to the cytoplasm, but that in the section shown in **b**, the spherule appears separated from the cytoplasm by two or more bounding membranes. **c** | Similar 50–70-nm spherular vesicles invaginated from the outer perinuclear ER membrane into the ER lumen, in a yeast cell expressing brome mosaic virus (BMV) replication factor 1a in the absence of other viral components. Indistinguishable spherules occur in cells expressing 1a and BMV 2a^{pol} in a 20/1 ratio and replicating BMV RNA3. Reproduced with permission from REF. 45 © (2002) Elsevier. **d** | Karmellae-like layering of the outer perinuclear ER membrane in cells expressing BMV 1a plus elevated levels of BMV 2a^{pol}, and replicating BMV RNA3. Note at top and bottom left that the ~60-nm intermembrane space is contiguous with the cytoplasm. Reproduced with permission from REF. 84 © (2004) National Academy of Sciences, USA.

regard, they parallel the foamy virus genus of retroviruses, which express Gag and Pol as independent proteins from separate mRNAs³⁴. Although expressed separately, direct *in vivo* interaction between the C-terminal 1a helicase domain and N-terminal sequences of nascent 2a^{pol} results in a 1a–2a^{pol} complex with a polarity that is reminiscent

Translational frameshift

A site-specific, programmed shift of some translating ribosomes from one reading frame to another, allowing a fraction of translation products to be extended in the new frame.

Translational readthrough

Programmed translation of some ribosomes through a termination codon, allowing a fraction of translation products to be extended beyond the normal stop site.

of orthoretrovirus Gag–Pol^{46–48} (FIG. 2). Moreover, whereas translation from separate mRNAs precludes regulation by frameshift or readthrough, BMV downregulates 2a^{pol} at translation initiation⁷⁵ and by degradation of 2a^{pol} that is not complexed with 1a (REF. 76). 1a–2a^{pol} interaction itself is downregulated by competing 1a–1a interaction⁵⁴ and 2a^{pol} phosphorylation⁷⁷. Therefore, like alphaviruses⁷⁸, bromoviruses have evolved several mechanisms to reduce polymerase accumulation and incorporation to achieve the retrovirus-like 1a to 2a^{pol} ratio of 25/1 in RNA-replication complexes⁴⁵.

Some other (+)RNA viruses, such as hepatitis C virus and picornaviruses, use polyprotein expression strategies that translate all viral ORFs, including polymerase, at equimolar levels. In at least some of these cases, only a fraction of polymerase sequences might be active owing to production of processing intermediates that lack polymerase sequences or activity, polymerase-active and -inactive conformers, polymerase sequestration in the nucleus and/or inclusion bodies, and other effects^{62,79}. Some retrotransposons that produce equimolar levels of Gag and Pol appear to use related strategies to regulate polymerase incorporation or activity^{80,81}.

Alternative membrane rearrangements. Whereas RNA replication by many (+)RNA viruses induces spherular membrane invaginations similar to those shown in FIG. 3a–c, some (+)RNA viruses induce alternative membrane rearrangements. In many cases, the topologies of these rearranged membranes remain under investigation and, as discussed below, some superficially distinct membrane structures might share underlying parallels in topology, assembly and function. Similarly, depending on the conditions, retrovirus Gag not only assembles normal capsids but also sheets, tubes and other structures⁸².

Flavivirus RNA replication localizes to packets in which an outer bounding membrane surrounds 50–100-nm vesicles that label with antibodies to viral RNA-replication proteins and dsRNA⁸³. These vesicle packets are similar to EM views of BMV spherules invaginated into the ER lumen⁸⁴ or nodavirus spherules invaginated into the lumen between the inner and outer mitochondrial membranes²², when sectioned perpendicular to the direction of invagination (FIG. 3b). BrUTP-labelled RNA synthesis by the related hepatitis C virus localizes to potentially similar clusters of ~85-nm vesicles surrounded by undulating membranes, termed

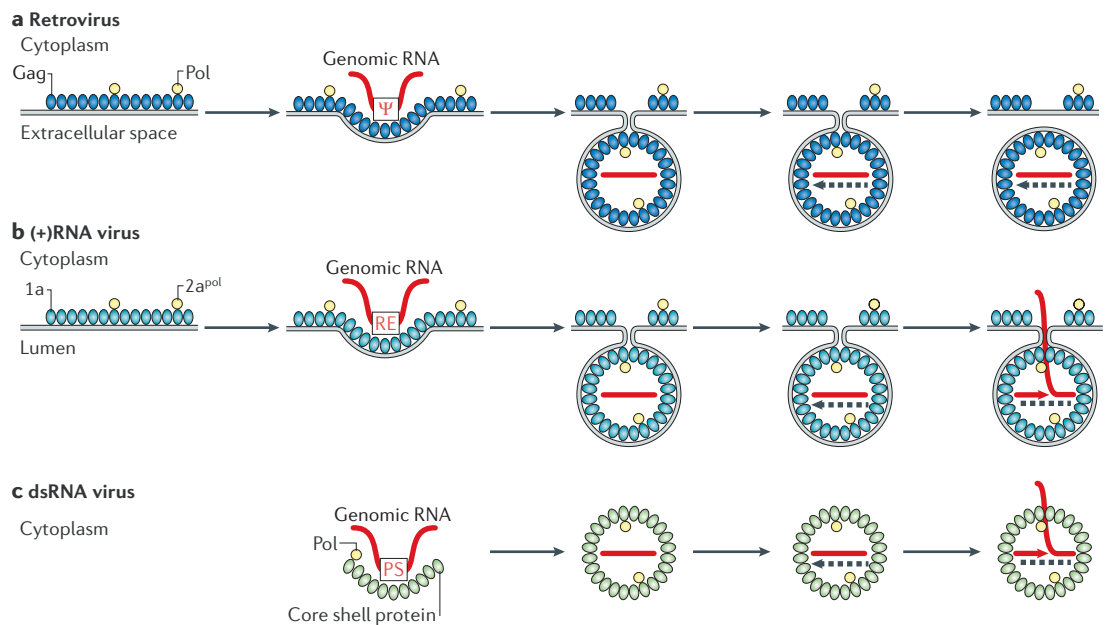


Figure 4 | Parallels between structure, assembly and function of retrovirus capsids, dsRNA-virus capsids and (+)RNA-virus RNA-replication complexes. Highly simplified schematics are shown in each case. **a** | Assembly of a retrovirus capsid includes the interaction of membrane-associated Gag and Gag–Pol. Gag-dependent genomic RNA encapsidation takes place through packaging signal Ψ, and this is followed by budding. To emphasize similarities with panels **b** and **c**, synthesis of negative-strand cDNA (dashed lines) is shown prior to budding, as occurs for foamy retroviruses. **b** | Assembly and function of a bromovirus RNA-replication complex at the outer endoplasmic-reticulum membrane includes interaction of membrane-associated 1a and 2a^{pol}. 1a-dependent genomic RNA encapsidation takes place through the recruitment element (RE) template recruitment signal. This is followed by synthesis and retention of negative-strand RNA (dashed black lines), and asymmetric synthesis and export of positive-strand progeny RNA (red lines), which for at least some positive-strand RNA ((+)RNA) viruses proceeds by a semi-conservative mechanism as shown¹⁴³. **c** | Assembly and function of the capsid core of a generalized double-stranded (ds)RNA virus includes encapsidation of genomic RNAs by a packaging signal (PS), synthesis and retention of negative-strand RNA (dashed black lines) and subsequent asymmetric synthesis and export of positive-strand progeny RNA (red lines). (+)RNA synthesis by dsRNA viruses can be either semi-conservative¹⁴⁴, as shown, or conservative^{90,145}.

the membranous web^{19,67}. Recently, models with strong parallels to bromovirus replication complexes have been proposed for RNA-replication complexes of hepatitis C virus^{62,85}.

RNA replication by picornaviruses, arteriviruses and coronaviruses occurs in conjunction with double-membrane vesicles, that is, vesicles bearing two closely appressed bounding membranes^{17,18,86}. Similar to spherules, arterivirus double-membrane vesicles are thought to form by invagination of appressed ER membranes¹⁷. Some EM sections of poliovirus double-membrane vesicles display a narrow neck that connects the inner and outer membranes to each other, and that also connects the vesicle interior to the cytoplasm⁸⁷, indicating possible genesis by invagination, continuing connection with the cytoplasm, or both.

Interconversion of membrane rearrangements. Further evidence linking seemingly distinct membrane rearrangements in RNA replication is that altering the relative levels and interactions of BMV replication factors 1a and 2a^{pol} shifts the associated membrane rearrangements between two dramatically distinct forms⁸⁴. Expressing 1a plus low levels of 2a^{pol} (1a/2a^{pol} ≈ 20) induces spherular replication complexes matching those of natural bromovirus^{24,25} and alphavirus²¹ infections (FIG. 3c). By contrast, expressing 1a plus higher levels of 2a^{pol} induces stacks of appressed double membranes that support RNA replication to levels as high as spherules⁸⁴ (FIG. 3d). The spaces between these double membrane layers parallel spherule interiors in being 50–60 nm wide, containing 1a and 2a^{pol}, and being directly connected to the cytoplasm. Therefore, BMV-induced spherules and double membrane layers seem to be functionally and topologically equivalent forms that are built from the same protein–protein and protein–membrane interactions, but in altered proportions.

Similar stacked, double-membrane ER layers and other ordered ER membrane arrays are induced by over-expression of membrane-associated picornavirus replication factors 2B and 2BC (REFS 68,88). Moreover, the double-membrane vesicles associated with picornavirus RNA replication, the spherule-bearing mitochondrial membranes associated with nodavirus RNA replication and the spherule-bearing chloroplast membranes associated with tymovirus RNA replication all cluster by interaction of surface membranes bearing viral replication factors^{22,23,63,89}. Therefore, a continuum of vesicle-forming and planar membrane interactions seems to be a normal part of RNA replication by many (+)RNA viruses.

(+)RNA virus and dsRNA virus parallels

Many of the above parallels between (+)RNA viruses and retroviruses extend to dsRNA viruses, which also sequester genomic RNA templates, viral polymerase and, often, RNA-capping functions in a protein shell for genome replication^{90,91} (FIG. 4c). Possible connections between certain (+)RNA and dsRNA viruses were recognized previously, based on conservation of polymerase^{5,92} and, in one case, on additional replication-factor sequences⁹³.

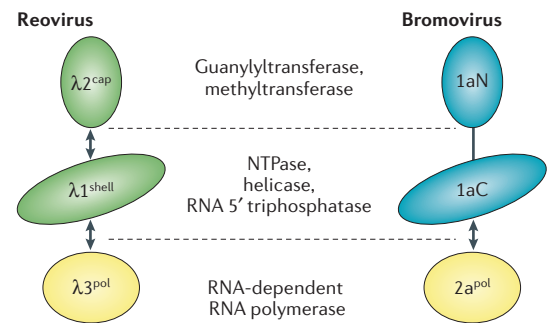


Figure 5 | Structure–function parallels between reovirus and bromovirus RNA-replication factors. The schematics illustrate similarities between interaction and function of reovirus core shell-forming protein $\lambda 1$, RNA-capping protein $\lambda 2$ and RNA-dependent polymerase $\lambda 3$, and the interactions and functions of the brome mosaic virus (BMV) 1a C-proximal NTPase/helicase domain (1aC), 1a N-proximal RNA-capping domain (1aN) and 2a^{pol} RNA-dependent RNA polymerase. The reovirus $\lambda 1$ – $\lambda 2$ – $\lambda 3$ interactions shown in the schematic occur at each of the twelve 5-fold axes of the reovirus core.

Similarities with Reoviridae cores. BMV RNA-replication factors and replication complexes share several similarities with the icosahedrally symmetric replicative cores of the dsRNA-virus family *Reoviridae* (FIG. 4). *Reoviridae* virions consist of one or more outer-protein shells that are shed during cell entry, surrounding an RNA-containing core that synthesizes RNA *in vivo* and *in vitro*⁹⁰. For the Orthoreovirus genus of the *Reoviridae*, the transcriptionally active, dsRNA-containing core is bound by an ~60-nm icosahedral shell made from 60 dimers of protein $\lambda 1$ (REF. 94). At each of the twelve 5-fold axes of the core, one copy of the $\lambda 3$ polymerase resides inside the shell⁹⁵, whereas on the shell exterior, a pentamer turret of the $\lambda 2$ capping proteins surrounds the 5-fold axis⁹⁴. The $\lambda 3$ polymerase copies the genomic dsRNA templates into new (+)RNA progeny, exporting the nascent RNAs to the cytoplasm through the $\lambda 2$ pentamer turret, which adds m⁷G caps to the 5' ends.

BMV 1a shows many parallels in function and protein–protein interactions with reovirus core proteins $\lambda 1$ and $\lambda 2$, implying potentially similar roles in RNA synthesis (FIG. 5). 1a parallels $\lambda 1$ as the self-interacting, high-copy-number inducer of the viral RNA-replication compartment, yielding the spherular BMV replication complexes. The 50–70-nm intra-membrane diameter of these spherules is similar to the reovirus $\lambda 1$ core-protein shell. The C-terminal 1a NTPase/helicase domain and its alphavirus homologues have NTPase, RNA 5'-triphosphatase and helicase activities^{96–99}, matching $\lambda 1$ (REFS 100,101). The 1a NTPase/helicase domain also anchors BMV RNA polymerase 2a^{pol} to the replication complex by direct interaction¹⁰², whereas $\lambda 1$ performs the same role for reovirus RNA polymerase $\lambda 3$ (REF. 95). The resulting twelve $\lambda 3$ polymerases per core are similar to the estimated 10–15 2a^{pol} per BMV RNA-replication complex⁴⁵. Continuing these parallels, $\lambda 1$ and the 1a NTPase/helicase domain each anchor RNA-capping

functions to the RNA-synthesis complex in the form of reovirus $\lambda 2$ protein and the BMV 1a N-terminal domain, respectively. $\lambda 2$ (REFS 103,104) and the 1a N-terminal domain^{42–44,105} both possess guanylyltransferase and methyltransferase activities that add m⁷G caps to the 5' ends of the (+)RNA products of their respective RNA-synthesis complexes.

RNA packaging, synthesis and export. Recent results indicate possible parallels between the recruitment of (+)RNA templates to BMV RNA-replication complexes and the packaging of (+)RNAs in dsRNA viruses. For dsRNA bacteriophage $\phi 6$, viral (+)RNAs are translocated into a pre-formed, empty core by a hexameric viral NTPase/helicase, P4 (REFS 106,107). Similarly, recruitment of BMV genomic RNAs from a membrane-bound, nuclease-sensitive state into the nuclease-resistant spherule replication complex, but not formation of the membrane-invaginated spherules themselves, is blocked by single amino-acid substitutions that inhibit the NTPase activity of the 1a NTPase/helicase domain⁹⁹. Also, like $\phi 6$ P4, the 1a-homologous NTPase/helicase domain of protein p126 of **tobacco mosaic virus** forms hexamers¹⁰⁸. For the *Reoviridae*, packaging viral (+)RNAs for replication also involves a viral protein with NTPase, RNA-binding and helix-destabilizing activities — the octameric NSP2 for the Rotavirus genus^{109,110} and, potentially, $\mu 2$ for the Orthoreovirus genus^{90,110,111}. Like $\phi 6$ P4 (REF. 106) and BMV 1a (REF. 112), $\mu 2$ also seems to be a co-factor for (+)RNA synthesis¹¹¹.

For dsRNA viruses, packaging of (+)RNAs is associated with, or followed by, (–)RNA synthesis, yielding dsRNAs that are protected in cores or core-like precursors^{90,91}. Similarly, BMV (–)RNA synthesis depends on 1a-mediated (+)RNA recruitment^{51,52}, and the resulting (–)RNA products are exclusively

retained in the nuclease-resistant, membrane-associated RNA-replication compartment⁴⁵. This retention in a virus-induced RNA-replication compartment explains the efficient, repetitive *in vivo* use of (–)RNAs as templates for (+)RNA synthesis, and the lack of (–)RNA exchange between RNA-replication complexes in the same cell¹¹³. Compartmentalization of RNA templates also seems to be important in protecting dsRNA-virus genomes and the potentially dsRNA-replication intermediates of (+)RNA viruses^{25,58} from dsRNA-induced host defences, including interferon responses and RNA interference^{90,91,114–116}.

A reovirus-like pathway for (+)RNA replication (FIG. 4b,c) would also explain the common early shutoff of (–)RNA synthesis in BMV and other (+)RNA viruses, while (+)RNA synthesis continues unabated^{112,117,118}, and the dependence of (–)RNA synthesis on continuing protein synthesis¹¹⁸. In such a model, (–)RNAs might be synthesized primarily during or immediately after replication-complex assembly from newly synthesized proteins, forming dsRNAs that would preferentially or exclusively be templates for (+)RNA synthesis in the resulting stable replication complexes. Replication-complex assembly and negative-strand synthesis seem to cease on exhaustion of a limiting host factor, possibly the target membrane for replication-complex formation^{15,118}. Pre-formed replication complexes, however, remain active in (+)RNA synthesis^{112,118}.

For both the *Reoviridae*⁹⁰ and the alphavirus-like superfamily^{119,120}, (–)RNAs are not capped. By contrast, (+)RNA products are modified with 5' m⁷G caps and exported to the cytoplasm from both dsRNA virus cores^{94,95,121} and BMV spherules^{43–45}. This and the parallels noted above between the capping functions and polymerase interactions of the responsible reovirus proteins and 1a suggest that the transcription/capping/export complexes at the 5-fold axes of dsRNA-virus cores might provide models for at least some aspects of the corresponding processes at the spherule necks of BMV and other (+)RNA-virus replication complexes.

Possible evolutionary relationships

The above results show that reverse-transcribing viruses, dsRNA viruses and many, if not all, (+)RNA viruses share central features of genome replication (FIG. 6). Most fundamentally, all replicate their genome through an RNA intermediate that also functions as an mRNA. In all three cases, viral proteins that are translated from these mRNAs capture the mRNA template with its polymerase in a multi-subunit core, sequestering viral RNA from competing processes such as translation and degradation, and sequestering polymerase from competing templates. The polymerase then copies the mRNA into a complementary template from which more mRNA is produced.

The variations on this theme that distinguish the three virus classes primarily relate to possessing an RNA-dependent RNA or DNA polymerase, and to deriving infectious virions from different intermediates in the common replication cycle. Orthoretroviruses, such as **HIV**, export the replicative core prior to copying

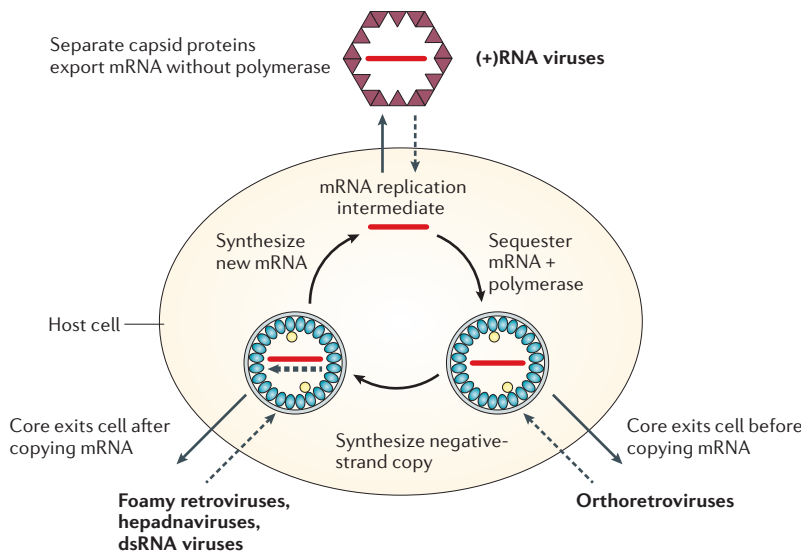


Figure 6 | Parallels and distinctions among the life cycles of reverse-transcribing viruses, (+)RNA viruses and dsRNA viruses. All three classes of virus share a similar replication cycle for their genomic RNA (central cyclical steps) but derive their infectious virions from different intermediates in that cycle (radial arrows). See text for further details. (+)RNA, positive-strand RNA; dsRNA, double-stranded RNA.

the mRNA intermediate (FIG. 6, bottom right). By contrast, foamy viruses, hepadnaviruses and dsRNA viruses export the replicative core after copying the mRNA intermediate (FIG. 6, bottom left). For (+)RNA viruses, the virion is separate from the replication complex, and capsid proteins that are not involved in RNA replication package and export the mRNA intermediate before it is sequestered with polymerase (FIG. 6, top). These distinct virion-assembly choices have important consequences, as noted in the introduction, but from some perspectives could be considered temporary extracellular excursions because, as soon as the next cell is infected, each virus re-enters the central, shared replication pathway.

Although no set of shared features can distinguish divergent from convergent evolution, the many parallels summarized here suggest that (+)RNA viruses, dsRNA viruses and reverse-transcribing viruses might have arisen from common ancestors. The transitions required for such evolution can be readily envisioned, and in some cases have precedents. RNA-dependent RNA and DNA polymerases, for example, have related structures^{122,123}, and a shift between the two has generally been postulated as the basis for the emergence of DNA-based biology from the RNA world⁹. In keeping with the potential for such transitions, BMV RNA-dependent RNA polymerase can copy DNA templates¹²⁴, and reverse transcriptase can, with single point mutations, incorporate ribonucleotide triphosphates (rNTPs) to produce RNA¹²⁵.

Established and emerging results further indicate that the intracellular RNA-replication complexes of (+)RNA viruses might evolve into infectious extracellular virions, similar to those of dsRNA or reverse-transcribing viruses, by relatively simple modifications. The spherule RNA-replication complexes of alphaviruses, for example, occur on endosomal membranes and, in lesser numbers, at the plasma membrane, where their appearance closely mimics budding virions⁵⁹. Such spherules are released into the medium at low frequency⁵⁹, which might explain the novel infectious particles released from cells replicating alphavirus derivatives with all natural virion proteins deleted and replaced by envelope protein G of **vesicular stomatitis virus**¹²⁶. As G protein is readily incorporated into many particles that bud from the plasma membrane and confers the ability to attach to, and fuse membranes with, many cells, incorporation of G protein into spherule membranes might confer infectivity by allowing released replication complexes or their RNAs to enter new cells.

Once acquired, infectivity might be enhanced and optimized through subsequently selected events. For retroviruses and other enveloped viruses, efficient virion budding requires recruiting functions from the host-cell multivesicular-body pathway⁵⁷. This host machinery can be recruited by incorporating into viral proteins any of a range of short protein-interaction motifs or L domains, which function in a largely position-independent manner⁵⁵. RNA-recombination events that are suitable to acquire such L domains, favourable envelope-protein genes or other relevant functions from cellular or other viral RNAs are rampant in (+)RNA viruses and retroviruses, representing a major force in virus evolution^{127–129}.

Additional pathways for evolutionary transitions between the intracellular RNA-replication complexes of (+)RNA viruses and dsRNA virions have recently been suggested^{6,130}. As the replication complexes of (+)RNA viruses are invariably membrane-associated, it is notable that virions of the Rotavirus genus of the dsRNA *Reoviridae* bud through membranes during assembly and exit from cells¹³¹. Moreover, rotavirus transmembrane protein nsP4, which mediates virion-core association with ER membranes, is required for correct assembly of the viroplasm in which core assembly and replicative RNA synthesis occur, indicating linkage of these processes with membranes^{132,133}. dsRNA bacteriophage ϕ 6 virions also become membrane-enveloped during exit¹³⁴.

Whereas most views of virus relationships have tended to emphasize the features and classes depicted in FIG. 1, it is intriguing that several alternative, orthogonal virus groupings are defined and mutually distinguished by features that are more strongly conserved across two or more of the classes in FIG. 1. Selected examples include the conservation of an unusual polymerase-sequence rearrangement between (+)RNA tetraviruses and dsRNA birnaviruses⁵, or the use of protein-primed genome replication by the (+)RNA picornaviruses, dsRNA birnaviruses and reverse-transcribing hepadnaviruses^{3,4,7}, in contrast to the use of tRNA-like elements by bromoviruses and retroviruses. Such relationships suggest that transitions between the virus classes depicted in FIG. 1 might have evolved on more than one occasion, and that links between viruses within a class might not always be as strong as links between classes.

Implications for virus control

The emergence of common underlying principles in the central replication processes of (+)RNA viruses, reverse-transcribing viruses and dsRNA viruses suggests that some of these shared features might provide useful targets for broader-spectrum or generalizable approaches for virus control. One approach might be to modulate membrane lipid composition, as recent results show that assembly and function of membrane-associated (+)RNA-virus replication complexes and retrovirus capsids are highly sensitive to the lipid composition of their target membranes, which in at least some cases can be manipulated by small-molecule therapeutics^{135–138}. Another general approach might be to interfere with oligomerization or function of oligomerizing replication factors such as retrovirus Gag and BMV 1a by dominant negative mutants^{43,99}, inhibition of required chaperones^{139,140}, targeted incorporation of destructive ligands or other approaches. Further interventions might be based on interfering with viral RNA interactions that are essential for genome packaging, replication and other steps, using nucleic-acid aptamers or other inhibitors¹⁴¹. Other approaches might interfere with the trafficking of viral replication factors and RNAs to their required intracellular sites of assembly¹⁴². Opportunities for such interference will continue to increase as further aspects of these processes are understood in greater detail.

Concluding remarks

An exciting aspect of current virology is that advancing results have not only continued to enrich our understanding of individual viruses, but also to reveal unifying principles that link many aspects of virus infection, replication and host interactions across a surprisingly wide breadth of virus–host systems. The resulting insights display a fundamental order within the vast and sometimes apparently chaotic diversity of known

viruses, with important ramifications for virus function and evolution. As the full extent of this tapestry of relations is still emerging, ongoing studies will continue to extend and refine the underlying mechanistic connections. In association with their mechanistic importance, the results should have a growing impact on our abilities to limit the continuing toll and emerging threat of viral diseases, and to develop the beneficial uses of viruses.

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Competing interests statement

The author declares no competing financial interests.

DATABASES

The following terms in this article are linked online to:
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 hepatitis C virus | HIV | poliovirus | RNA1 | RNA2 | RNA3 | tobacco mosaic virus | vesicular stomatitis virus
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