

Parvoviruses: Small Does Not Mean Simple

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Abstract

Parvoviruses are small, rugged, nonenveloped protein particles containing a linear, nonpermuted, single-stranded DNA genome of ~5 kb. Their limited coding potential requires optimal adaptation to the environment of particular host cells, where entry is mediated by a variable program of capsid dynamics, ultimately leading to genome ejection from intact particles within the host nucleus. Genomes are amplified by a continuous unidirectional strand-displacement mechanism, a linear adaptation of rolling circle replication that relies on the repeated folding and unfolding of small hairpin telomeres to reorient the advancing fork. Progeny genomes are propelled by the viral helicase into the preformed capsid via a pore at one of its icosahedral fivefold axes. Here we explore how the fine-tuning of this unique replication system and the mechanics that regulate opening and closing of the capsid fivefold portals have evolved in different viral lineages to create a remarkably complex spectrum of phenotypes.

HALLMARKS OF PARVOVIRAL INFECTIONS

Homotelomeric:

having similar hairpin telomeres at each end of the genome that are part of inverted terminal repeats

Heterotelomeric:

having hairpin telomeres at the two ends of the genome that differ in size, sequence, and potential secondary structure

Whereas large viruses can devote considerable genetic capital to individual steps in infection, evolution has optimized and focused the coding and replicative potential of small viruses, greatly constraining their courses of productive infection. Members of the family Parvoviridae are ubiquitous, physically minute, and genetically compact viruses that contain a linear, single-stranded DNA genome of ~5 kb, which encodes just two genes, a genetic architecture that is, to our knowledge, unique in the biosphere. Necessarily, the passive strategies these viruses employ to mediate some steps in infection differ profoundly from those used by their larger cousins, which face the world equipped with accessory proteins and functional duplex transcription templates. To overcome this limitation, one well-studied group of parvoviruses, the adeno-associated viruses (AAVs), relies on coinfection with a member of various double-stranded DNA virus families, and many steps in the establishment of infection are delegated to the helper virus (1–3).

However, most parvoviruses achieve autonomous, helper-virus-independent replication in a limited range of differentiated cell types to which they are optimally adapted (2–4). Unlike almost all other DNA viruses, parvoviruses lack a mechanism that can drive noncycling host cells into S phase and thus access the cellular replication machinery needed to synthesize the first complementary viral strands (5). Because their incoming single-stranded genomes do not support transcription, infection is restricted to dividing cell populations that embark on S phase under their own cell cycle control, rendering fetal or neonatal hosts particularly vulnerable. Probably for the same reason, parvoviruses are not oncogenic—indeed, some are selectively oncotropic and oncolytic, particularly outside their natural host species (6). They are also masters of stealth, evading detection or DNA modification by cellular surveillance systems that would otherwise delay S phase or “repair,” and hence inactivate, the viral genome.

In S phase, parvoviruses rapidly usurp the cellular machinery, employing a unique replication strategy that is a linear adaptation of the rolling circle mechanisms used by many small circular replicons. Replication generates concatemeric duplex intermediates via a unidirectional strand-displacement mechanism, from which the ends of progeny genomes are excised by a virally coded site-specific single-strand endonuclease/helicase. For homotelomeric parvoviruses, in which the two genomic termini are identical, this process is symmetrical, and strands of both polarities are displaced and packaged; however, if the two termini are substantially different—i.e., heterotelomeric—they are resolved at different rates, by different mechanisms, typically leading to the displacement and encapsidation of strands that are predominantly negative sense (5). These are packaged into small (~280-Å-diameter), preformed $T = 1$ protein capsids via a cylindrical pore at an icosahedral fivefold axis (7, 8), driven by the viral helicase (9). Resulting particles are exceptionally robust and long lived, allowing their widespread dissemination through host populations and the environment (10, 11). Despite their stability, they undergo a variable series of conformational shifts that allow the genome to be packaged and subsequently uncoated, and that ultimately mediate transport of intact particles into host cell nuclei and, in some instances, progeny virions out of the cell (12, 13). In this review we correlate incremental phylogenetic shifts in the structure and dynamics of the capsid portals with differences in the replication bias and lifestyle of these minute viruses.

THE FAMILY: DIVERSITY AMONG THE PARVOVIRIDAE

Members of the family Parvoviridae infect a broad range of hosts, as reflected by their division into two subfamilies, Parvovirinae and Densovirinae, whose members infect vertebrate and arthropod hosts, respectively. In this article we focus exclusively on members of the Parvovirinae, which have been studied in greater detail to date. Knowledge of viral diversity in this subfamily has

greatly increased in recent years, in part due to virus discovery approaches that have identified many new genomes and even entirely new lineages. To take these into account, the taxonomy was recently expanded and rationalized by the International Committee on Virus Taxonomy (14, 15). These revisions identify many new viruses within the subfamily Parvovirinae, which is now divided into eight genera. Five preexisting genera, previously called *Amdovirus*, *Bocavirus*, *Dependovirus*, *Erythrovirus*, and *Parvovirus*, are now called *Amdoparvovirus*, *Bocaparvovirus*, *Dependoparvovirus*, *Erythroparvovirus*, and *Protoparvovirus* to flag their family affiliation and enhance taxon clarity. To these have been added three new genera, *Aveparvovirus*, *Copiparvovirus*, and *Tetraparvovirus*, as diagrammed in **Supplemental Figure 1** (14). Two important new groups of human viruses are recognized. The first comprises human bocaviruses 1–4 (HBoV1–4) in the genus *Bocaparvovirus*, which cause widespread respiratory and gastrointestinal tract infections (16, 17). The second includes human parvovirus 4 (PARV4). In Europe and North America, this virus is found predominantly in populations at risk for blood-borne diseases, suggesting that transmission occurs via a parenteral route. However, it shows much higher incidence rates in some geographic locations, where it likely uses an alternative route of transmission. PARV4 is the founding species of the new *Tetraparvovirus* genus, other members of which infect nonhuman primates, domestic ungulates, and Old World bats (18, 19).

Although genus demarcations are determined primarily by protein sequence identity, important viral characteristics also segregate between taxa and are intrinsic to the phylogeny. Viruses from different genera often employ widely disparate solutions to the same steps in infection. For example, they typically encode radically different small ancillary proteins, exhibit distinctive telomere structures, and use polyadenylation signals, suboptimal start codons, alternative splicing patterns, collinear open reading frames, and different numbers of promoters to differentially access coding potential within the two viral gene cassettes (20–25). Due to space constraints, examples cited in this article focus predominantly on minute virus of mice (MVM), from the genus *Protoparvovirus*, one of the most extensively studied autonomously replicating parvoviruses.

Replicative form (RF) DNA: duplex monomeric, dimeric, and tetrameric arrangements of the viral genome in palindromic concatemers

Flip and flop: inverted complementary forms of a single imperfect palindromic sequence

THE GENOME: TWO GENE CASSETTES FLANKED BY HAIRPIN TELOMERES

Parvoviruses are among the smallest known viruses. They contain one copy of a nonpermuted, linear, single-stranded DNA genome, ~5 kb in length, which, in the subfamily Parvovirinae, is monosense with respect to transcription. The coding sequence is flanked by short (116–385-base) imperfect palindromes that can fold into hairpin structures. Individually, these telomeres give rise to viral replication origins in replicative form (RF) DNA, and together they contain most of the *cis*-acting information required for both viral DNA replication and packaging. Palindromic elements that can alternate between intra- and interstrand base-pairing occur in many rolling circle origins, but in the parvoviruses these have been duplicated and expanded into multifunctional terminal structures that alternately fold and unfold during replication. Parvoviral hairpins vary in size, sequence, and secondary structure among genera but are quite conserved within a particular genus. As detailed in **Supplemental Figures 1** and **2**, all members of a genus are either homotelomeric or heterotelomeric, which markedly influences their biology. Homotelomeric viruses have inverted terminal repeat sequences that extend beyond the hairpin; not only do these viruses package equal numbers of plus and minus strands—in separate particles—but each telomere exists as a 50:50 mix of flip and flop orientations, where flip is the inverted complement of flop. Some homotelomeric viruses, most notably AAV2, can establish latency by integrating into host genomes, either site specifically (26) or nonspecifically (27). Because they contain recurring sequence elements, the telomeres of these genomes can undergo inter- and intragenomic recombination to produce

Replication initiators:

proteins that introduce single-strand nicks into duplex origins, exposing base-paired 3'-hydroxyls that prime assembly of new replication forks

Superfamily 3 (SF3) helicases:

a subfamily of AAA+ helicases found only in viral initiator proteins

concatemeric duplex circles, which are very stable and may represent the major form of the genome in postmitotic cells and latent infections (1, 2, 28).

In contrast, viruses with heterotelomeric genomes generally show packaging bias, with minus strands encapsidated in preference to plus strands by a 10- to 100-fold margin. This results from differences in the efficiency of their two DNA replication origins, rather than any strand-specific packaging sequence (29). By convention, the 5' and 3' ends of plus strands are designated as the left and right ends of the genome, respectively. As in their homotelomeric cousins, the right-hand hairpins of heterotelomeric viruses exist as a 50:50 mix of flip and flop sequence orientations, reflecting their processing by a similar terminal resolution nicking mechanism, but their left-hand ends predominate in the flip orientation, a hallmark of their generation by a junction resolution strategy (30). Less is known about how heterotelomeric viruses survive in noncycling or postmitotic cells, but one possibility is that they persist as intact virions in the nucleus, in what has been termed cryptic infection.

The terminal hairpins bracket a single-stranded coding region of 4.5–5 kb that contains two major gene cassettes, as diagrammed in **Supplemental Figure 2**. Invariably, the left half of the genome encodes a small number of nonstructural (NS) proteins essential for replication, and the right half encodes two or more N-terminal variants of a single capsid protein. Expression strategies vary significantly, with members of the *Protoparvovirus* and *Dependoparvovirus* genera using two to three separate transcriptional promoters, as illustrated for MVM in **Supplemental Figure 3**. In contrast, members of the *Amdoparvovirus*, *Erythroparvovirus*, and *Bocaparvovirus* genera express all genes from a single promoter and supplement alternative splicing with genus-specific mechanisms to conditionally override a central polyadenylation signal that otherwise blocks access to VP-coding sequences (20–25).

Nonstructural Proteins: A Replication Initiator plus Variable Ancillary Proteins

The NS gene encodes a large (~70–100 kDa), multidomain replication initiator protein, called NS1 in most parvoviruses but Rep in the AAVs. This multifunctional protein has overlapping N-terminal site-specific duplex DNA-recognition and site- and strand-specific endonuclease domains, with cation-binding and active-site tyrosine protein motifs that resemble those of other rolling circle nucleases, linked to a central superfamily 3 (SF3) helicase domain with 3'-to-5' processivity (2, 31–35). In addition to their central role in DNA replication, some NS1 proteins serve multiple additional functions, most notably *trans*-activating viral gene expression after binding site specifically upstream of a transcriptional promoter (36–38). These proteins are commonly monomeric in solution but assemble into multiple oligomeric complexes when interacting with DNA (39). In MVM, and probably other protoparvoviruses, a significant fraction of the NS1 is complexed site specifically with intracellular duplex viral DNA, via a series of slightly disparate recognition sites that are dispersed throughout the genome. These promote binding but not nicking (40), and together generate a distinctive pseudochromatin structure. In addition, ~0.5% of the NS1 in MVM-infected cells is covalently attached to the 5' ends of RF and single-stranded progeny DNA (41).

To supplement the NS and VP proteins, each virus expresses a small number of genus-specific ancillary proteins (see **Supplemental Figure 2**). Members of the genera *Amdoparvovirus* and *Protoparvovirus* encode essential ancillary NS2 and small alternatively translated (SAT) proteins (42). The amdoparvovirus Aleutian mink disease virus (AMDV) also expresses an overlapping essential NS3 molecule of unknown function (43). MVM NS2 exists as three isoforms, P, Y and L, which differ in their C-terminal hexapeptide sequences and have multiple functions that are mostly indirect, mediated by interactions with host proteins. Although NS2 is not essential in

many transformed human cell lines, absence of the P isoform in MVM-infected murine cells leads to rapid paralysis of the infectious cycle soon after the start of viral DNA synthesis (44, 45). NS2 molecules typically have a half-life of around 1 h and associate in vivo with proteins from the cellular 14-3-3 family (46) and with the nuclear export factor CRM1 (47). NS2:CRM1 interactions exhibit supraphysiological affinity and are essential for efficient virion export prior to cell lysis (48–50).

The SAT protein is encoded within the capsid gene and is expressed late, from the same mRNA as VP2. It affects the rate at which virus spreads through culture, although the underlying mechanism remains obscure (42). The AAVs encode an assembly-activating protein (AAP) in a position and by a mechanism analogous to those of SAT (42, 51). AAP targets newly synthesized capsid proteins to the nucleolus, where it promotes capsid assembly via interactions with VP C-terminal peptides (51). Both SAT and AAP contain essential N-terminal hydrophobic domains but otherwise exhibit little functional or sequence homology. Parvovirus B19, a member of the genus *Erythroparvovirus*, encodes two ancillary proteins: an 11-kDa protein that is essential for infectivity, playing a role in virion production and trafficking (52) and in inducing apoptosis (53), and a 7.5-kDa protein of unknown function. In contrast, all members of the genus *Bocaparvovirus* encode an abundant nuclear phosphoprotein, NP1, which has a half-life of 6–8 h and is required for efficient duplex DNA amplification (25, 54, 55). NP1 is functionally distinct because it also influences RNA processing, promoting read-through of the internal polyadenylation signal and thus allowing capsid protein expression (25). These disparate supplementary proteins thus reflect the great diversity of parvoviral adaptation to individual host environments.

Phospholipase A2 (PLA2): enzyme that hydrolyzes the *sn*-2 acyl bond of phospholipids, releasing arachidonic acid and lysophospholipids

A Nested Set of Capsid Proteins

At least two size variants of the capsid gene are expressed, which share a common C-terminal core sequence of 520–560 amino acids that forms the capsid shell but have different-length N-terminal extensions. The longest protein, VP1 (~80–100 kDa), is present at 5 to 10 copies per virion and is essential for cell entry. In most viruses (except those in the genus *Amdoparvovirus*), the N-terminal VP1-specific region (VP1^{SR}) contains an essential phospholipase A2 (PLA2) enzymatic domain (56). Initially sequestered in mature virions, this domain becomes exposed at the particle surface during cell entry and refolds into an atypical, tethered, broad-specificity enzyme (57, 58). For AAV2 and MVM, PLA2 exposure occurs in the endosomal compartment, where its activity (59) is required to mediate virion transfer across the endosomal bilayer (60, 61), as illustrated in **Figure 1**. In contrast, the PLA2 domain of the B19 VP1^{SR} is prepositioned at the virion surface but masked by an additional, genus-specific, N-terminal peptide extension (62). Recent reports indicate that binding to its primary cell surface receptor, the P antigen globoside, induces a structural rearrangement in B19 VP1 that exposes its PLA2 activity and allows the peptide extension to bind a second cell surface molecule. The identity of this second receptor remains uncertain, but it appears to be cell-lineage specific and is essential for infectious entry, indicating a novel role for this VP1^{SR} in receptor-mediated host range (62). Irrespective of mechanism, the VP1^{SR} becomes exposed by the time the virion enters the cytoplasm (63), displaying clusters of basic amino acids and other elements that are required for transcytotic trafficking and nuclear entry (1, 63–65). Whether entry occurs through nuclear pores or via transient local disruption of the nuclear membrane is the subject of much current interest (66, 67).

Capsids are typically assembled from a few (5 to 10) copies of VP1 and 50 to 55 copies of various N-terminally truncated VP polypeptides. The domains contained in their N termini (and the numbering schemes) vary among genera. Thus, AAV particles contain ~5 copies of VP1

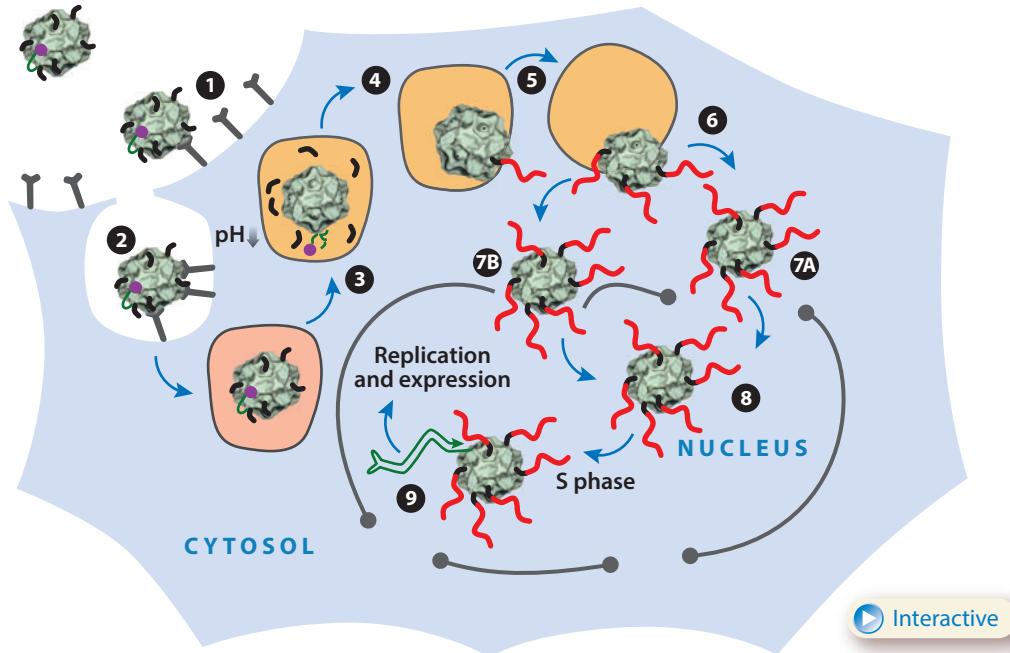


Figure 1

Cell entry pathway for minute virus of mice (MVM). Extended Y shapes represent cell surface receptors; colored lines associated with virus particles represent VP2 N termini (black), the VP1-specific region (red), and the viral genome (green). The purple spheres represent NS1. Although six VP1 N termini are shown as extruded, the actual number is not known and could vary between one and ten. The gated portals to the nuclear space indicate nuclear pores, whereas the ungated gaps indicate alternative access by disruption of the nuclear envelope. Hover over each number for an explanation of the corresponding step.

(87 kDa), ~5 copies of VP2 (73 kDa), and ~50 copies of the shortest form, VP3 (61 kDa), whereas MVM virions assemble from ~10 copies of VP1 (83 kDa) and ~50 copies of VP2 (63 kDa), as indicated in **Figure 2**. MVM capsid precursors are transported into the nucleus as trimers, via a structure-dependent, nonconventional nuclear localization motif (68). Two forms of trimer have been characterized, a homotrimer of VP2 and a less abundant heterotrimer that contains one VP1 complexed with two VP2 molecules (69). Assembly into empty MVM capsids occurs in the nucleus. Similarly, AAV VP proteins assemble into oligomers in the cytoplasm but do not form capsids. Unassembled precursors with sedimentation values of 10–15S have been identified, which may represent trimers and/or pentamers of the various VP proteins (1). Despite the strong evidence for early AAP-mediated assembly of AAV capsids in nucleoli (51, 70), this has yet to be documented for other viruses. Capsid formation sequesters all preexisting nuclear localization signals inside the particle, rendering the assembled capsid transport neutral.

THE FIVEFOLD CYLINDER: A DYNAMIC CAPSID PORTAL

Though exceptionally robust, parvoviral particles are also metastable, undergoing a program of conformational shifts that span the viral life cycle. Atomic or near-atomic structures of full and empty virions and recombinant virus-like particles are currently available for members of five genera (1, 3, 71–76). In each case, the modeled $T = 1$ icosahedral capsid contains 60 highly interdigitated copies of the VP core domain. This sequence establishes two-, three- and fivefold

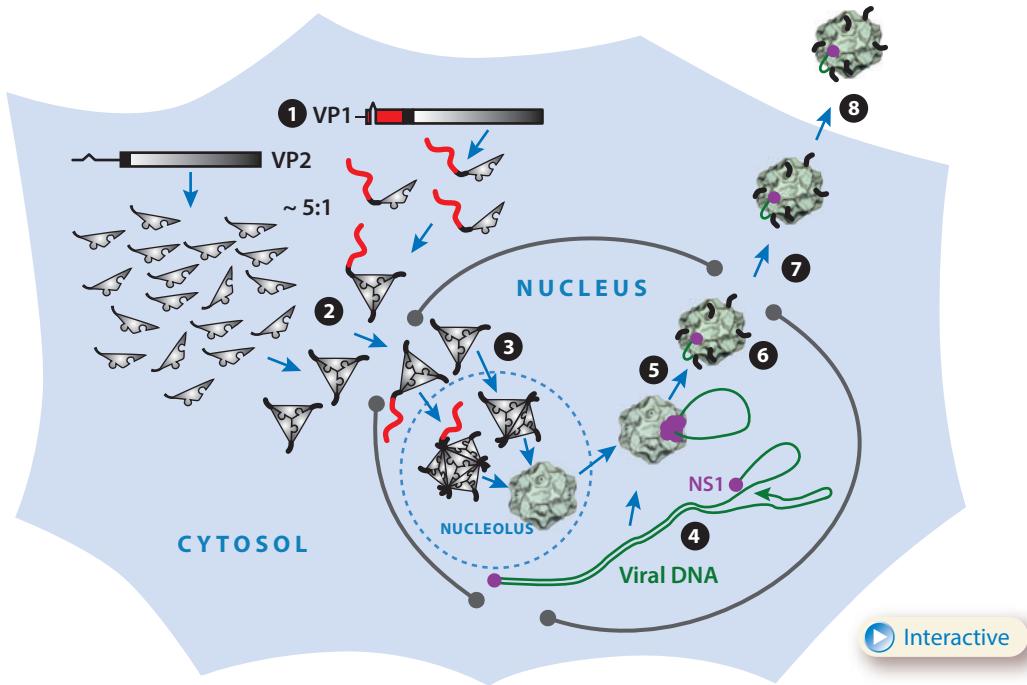


Figure 2

Virion assembly pathway for minute virus of mice (MVM). Long horizontal rectangles represent primary VP gene transcripts, and jigsaw triangles represent the folded core common to all VP molecules. Colored lines associated with virus particles represent VP2 N termini (black), the VP1-specific region (red), and the viral genome (green); purple spheres represent NS1. Hover over each number for an explanation of the corresponding step.

symmetry-related interactions, which define the icosahedral asymmetric unit of the particle and create its characteristic topological features, illustrated for MVM in **Figure 3**. These include depressions at the 30 twofold axes, which in MVM mediate tissue host range via glycan interactions (3, 4); elevations surrounding the 20 threefold axes, which can be involved in both glycan and protein receptor-mediated host range interactions (1–4); and cylindrical projections at the 12 fivefold axes, which are surrounded by a conserved circular, canyon-like depression of undefined function. The fivefold cylinders are formed by the juxtaposition of potentially flexible, antiparallel β -hairpins from each of the five symmetry-related VPs and enclose a narrow central channel that connects the interior of the capsid to its outer surface. Despite their apparently small diameter ($>8 \text{ \AA}$), structural, biochemical and genetic evidence accumulated for members of the *Protoparvovirus* and *Dependoparvovirus* genera implicates these pores as the entry and exit portals for the viral genome and for the sequential externalization of VP N termini (7, 8, 60, 77). In B19 virions, however, the pores do not mediate postassembly peptide extrusion (78, 79), suggesting that their primordial role is to allow genome translocation.

Three Genus-Specific Patterns of Cylinder Dynamics

Structural understanding of the VP N termini remains elusive. In X-ray reconstructions, the first ordered residue is invariably located inside the capsid, at the base of the fivefold pore, leaving unresolved VP1 sequences of 180–245 residues. This “unordered” region is divided into distinct

Fivefold cylinders:
capsid projections surrounding each fivefold axis with a central pore that penetrates to the capsid interior

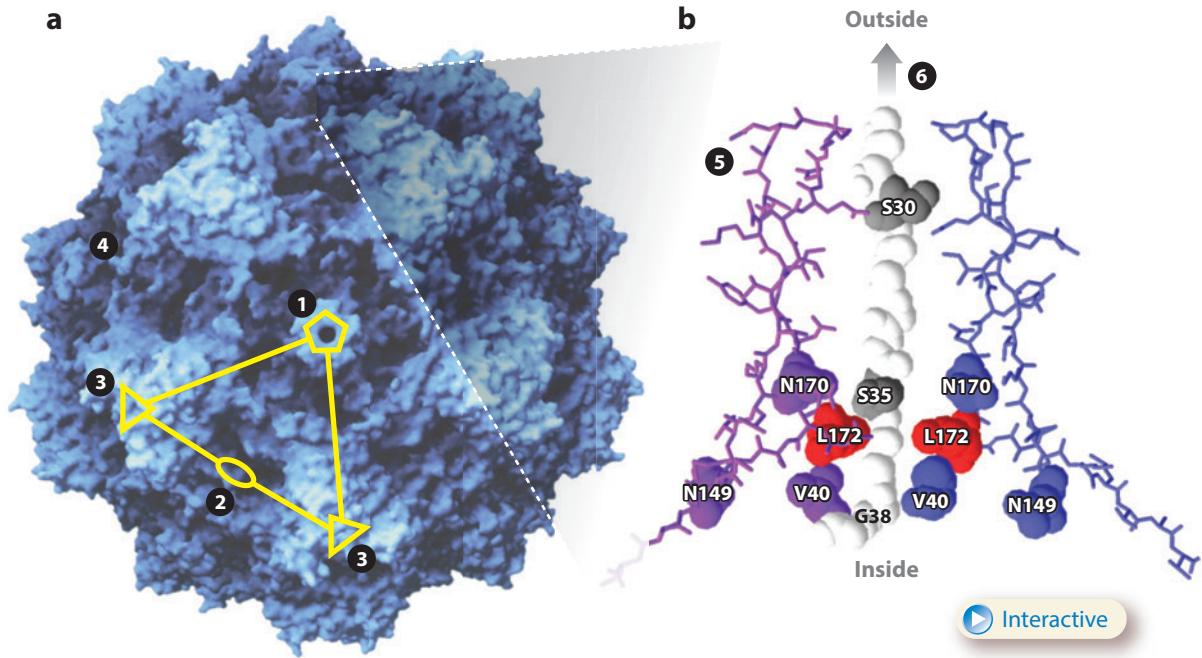


Figure 3

Surface topography of minute virus of mice (MVM) and the structure of the fivefold cylinder. (a) Depth-cued, space-filling model of the MVM capsid centered on one of the 12 fivefold symmetry axes, with the asymmetric unit delineated by yellow lines. The image of the virion exterior was generated from PDB 1MVM using the program GRASP, and was kindly provided by Dr. Mavis Agbandje-McKenna and Dr. Michael Rossmann. (b) Expanded stick model of the virion cylinder wall, showing two of five symmetry-related β -strand hairpins surrounding a space-filling model of the resident poly(G) tract. The image was generated from PDB 1MVM using Swiss PDB Viewer. Hover over each number for an explanation of the corresponding component.

domains, the most C-terminal of which is a glycine-rich sequence, shared by all VPs, that becomes threaded through the capsid shell. Cryo-electron microscopy reconstruction studies indicate that in both B19 virions and empty particles, this domain is positioned between neighboring fivefold-related VPs and emerges on the particle surface next to the cylinder, where the observed density suggests most or all of the VP N termini are located (78). However, in MVM virions, but not in empty particles, the first 10 amino acids from a single copy of the glycine sequence (VP2 G37–G28) can be modeled into submolar density that occupies the central pore of the cylinder, as shown in Figure 3. Accessibility studies indicate that in infectious particles this sequence belongs to the N terminus of VP2, but not of VP1, which remains sequestered within the capsid. In MVM, VP2 N termini contain a nuclear export signal (80), and their extrusion during genome packaging (81) effectively converts the trafficking-neutral capsid into a nuclear export–competent virion. The ~50 AAV VP3s are too short to project beyond the pore, suggesting that these viruses do not employ a similar early export pathway.

AAV and MVM VP1 N termini are initially positioned inside the particle but are translocated to the virion surface during cell entry. AAV cylinders are somewhat wider and shorter than those of MVM, but they have similar structures and the amino acids lining the pore are often identical. However, cylinder dynamics are surprisingly different between the two viruses. During AAV cell entry, and in vitro following substantial heating (60°C for ~30 min), the N termini of

VP1, and perhaps also VP2, are irreversibly extruded, potentially following a single structural rearrangement (7, 57). Significantly, mutation of critical residues lining the AAV pore prevents this rearrangement and renders the particle noninfectious (7). MVM cylinder dynamics are substantially more complex than those of their dependoparvovirus cousins. All MVM N-terminal peptides are sequestered in empty particles, but a subset of the VP2 termini become externalized early during genome encapsidation, and virions are released from the infected cell in this form (81). However, both in the extracellular environment and during cell entry, the exposed N termini are highly susceptible to proteolytic cleavage, which removes ~25 amino acids and converts VP2 to a form called VP3. Because X-ray structures show only one polyglycine tract threaded through the cylinder, as illustrated in **Figure 3b**, it is significant that ~90% of the ~50 VP2 termini eventually become surface exposed and cleaved. Mutation of amino acids lining the MVM pore effectively destabilizes the cylinder after VP2 cleavage, rather than rendering it inflexible, as in AAV. X-ray studies of MVM also indicate that VP2 cleavage allows the polyglycine tracts of cleaved termini to be retracted into the capsid shell, where they assume additional icosahedral ordering (82). These observations suggest a whack-a-mole model for MVM cylinder dynamics, in which proteolysis of one cohort of VP2 external domains transiently releases the cylinders from VP2-imposed stability constraints, allowing them to flex back into the canyon, widening the pore and allowing both retraction of the truncated polyglycine tract and extrusion of a fresh wave of VP2 N termini, complete with their bulky N-terminal side chains. In vitro cleavage of these termini proceeds to near completion at temperatures well below 37°C and substantially lowers the thermal energy required to induce subsequent exposure of the VP1^{SR} (83). It is likely, then, that this is the normal sequence of events *in vivo*, and that the cylinders have evolved to allow most VP2 N termini, and their associated nuclear exit signals, to be externalized and removed prior to, or during, early stages in host cell entry.

As illustrated in **Figure 3b**, the side chains of five symmetry-related leucine residues at VP2 position 172 constrict the MVM pore at the base of the cylinder, creating its narrowest (8-Å) internal diameter. Mutation of this amino acid to threonine generates a virion that can protect its VP1 N termini only if a sufficient number of VP2 N termini remain intact (83). However, when these are proteolysed, VP1 N termini are rapidly exposed and cleaved *in vitro*, even at low temperature. These observations thus support a central element in the whack-a-mole model, namely that an important function of the exposed VP2 N termini in DNA-containing particles is to stabilize the cylinders. If VP2-intact L172T virions are used to infect cells, their genomes are rapidly released in the endosomal entry pathway in both a 3'-to-5' and 5'-to-3' direction, apparently in a series of discrete pulses (84). Because genomes are packaged 3'-to-5', the observed 5'-to-3' release of DNA by this mutant appears to represent collapse of the unique packaging vertex and suggests that the ring of symmetry-related L172 side chains acts as an essential molecular ratchet, preventing the genome from reversing out of the cylinder after packaging.

Genome release in the reverse, 3'-to-5' direction is observed both for L172T and for virions carrying V40A, N149A, and N170A mutations, positioned around the base of the pore as shown in **Figure 2** (84). This polarity is significant because it recapitulates an uncoating reaction observed *in vitro* for wild-type virions induced by exposure to 37°C following incubation with chelating agents. Uncoating leaves the capsids intact and still interlaced with genomic 5' sequences (85). Cylinder-mutant studies (84) thus confirm that, both *in vivo* and *in vitro*, MVM cylinders can mediate progressive 3'-to-5' genome release, suggesting that this morphogenetic pattern is embedded in the virion and that a cellular trigger mechanism may exist *in vivo* to initiate uncoating. Significantly, packaging occurs in the same 3'-to-5' direction, also through a fivefold cylinder, and leaves the 5' end of the genome threaded through the entry portal, making it highly unlikely that DNA entry and exit can occur via the same vertex.

Rolling hairpin replication (RHR):

linear adaptation of RCR in which hairpin telomeres fold and unfold to reverse a unidirectional fork

Rolling circle replication (RCR):

nicking, synthesis, and rejoicing of single-strand circles displaced from a circular duplex template

To date, cylinder dynamics have been examined in members of only three of the eight Parvovirinae genera, so additional strategies or subtleties of adaptation may still emerge. It will be important to establish whether all parvoviruses encapsidate and eject their genomes in similar ways, how or when the unique vertices are selected, and what triggers their deployment. The relative advantages of positioning a capped form of the PLA2 domain at the virion surface, as seen for B19, versus retaining it deep inside the virion, remain uncertain, and it may be significant that initial studies indicate that the enzymatic activity of B19 PLA2 is exceptionally weak (59). Because amdoparvoviruses lack this domain entirely, alternate entry mechanisms must exist, suggesting an evolutionary path in which an ancestral virus first captured a rudimentary PLA2 activity. Although this assisted bilayer penetration, it may have presented other challenges *in vivo*, leading to its initial adoption as a weak enzyme. Subsequent changes in cylinder dynamics that resulted in its more effective sequestration within the particle, until activated during cell entry, could then have allowed selection of a more enzymatically active form. In this scenario, MVM would represent a further evolutionary step, with its complex cylinder dynamics evolving to allow the timed exposure of additional peptides carrying nuclear export signals, which would drive release of progeny virions prior to cell lysis. Parvoviruses exhibit high mutation rates (86–88), likely due to a combination of their reliance on single-stranded genomes and their unique replication strategy, so that radical adaptations could arise and become stabilized quite readily.

A UNIQUE MODE OF VIRAL DNA REPLICATION

During productive infection, incoming virions appear to enter the nucleus intact (13), but for MVM, the genomes then remain inert until S phase (5). Whether or not genomes remain sequestered within the particle throughout this early prereplication phase is uncertain, but such retention could explain why host DNA damage surveillance mechanisms are not activated, thus allowing cells to progress naturally into S phase. It may also explain how the single-stranded DNA and ornate genomic termini evade host “repair,” which would otherwise inactivate the virus. The presence of the capsid in the nucleus may allow it to play additional, novel roles in establishing infection, either before or after uncoating, perhaps by directing intranuclear genome location or recruiting essential cellular factors.

Once the host embarks on cellular DNA replication, uncoated viral genomes are converted into duplex RF molecules by a unidirectional leading-strand cellular fork, primed from the viral 3' hairpin telomere. These early RF molecules support viral gene expression, allowing the virus to rapidly usurp control of cell metabolism. Surprisingly, the two “ears” of the MVM left-hand hairpin, diagrammed in **Supplemental Figure 4**, are absolutely required for the establishment of these initial transcription complexes. Single-ear mutants fail to express their NS genes, although their genomes reach the nucleus, uncoat, and convert to RF molecules that can be *trans*-activated for both transcription and replication by NS1 provided in *trans* (89). Whether or not the incoming viral capsids play a role in this process remains an intriguing question.

During the S phase following infection, viral genomes are amplified via a single-strand displacement mechanism called rolling hairpin replication (RHR) that ensures duplication of both termini (90). RHR is a linear adaptation of the rolling circle replication (RCR) mechanisms used by many small circular replicons and is mediated predominantly by a subset of the synthetic machinery of the host, supplemented and orchestrated by the viral initiator protein, NS1. RHR is efficient and flexible but relies on a unidirectional fork prone to strand switching and displaces long stretches of single-stranded DNA. Although NS1 retains the site- and strand-specific *trans*-esterification activity characteristic of all RCR initiator endonucleases, it has lost the subsequent joining function that these use to circularize progeny genomes. Instead, the nickase is left covalently attached to

the 5' ends of all viral DNAs via its active-site tyrosine, where it persists throughout replication, packaging, and virion release. Replication is associated with structural rearrangement and enlargement of host cell nuclei (91, 92), and progression through the cell cycle is suspended, leaving cells actively synthesizing viral DNA until subsequent cell death results in the release of a final burst of progeny virus, a process that can take several days (5). In all viruses studied to date, activation of one of the DNA damage–responsive PI3-kinase-like kinases is essential for productive infection, possibly because these pathways provide opportunities for suppressing cellular, but not viral, DNA replication (93–95). Overall, parvoviruses evoke a wide and variable range of host DNA damage responses, some of which are deleterious and are evaded although others are co-opted for effective progeny virus production in this modified nuclear environment (5, 96–99).

PI-3-kinase-like kinases: kinases central to DNA damage responses: ataxia telangiectasia mutated (ATM), ATM and Rad3-related (ATR), and DNA-dependent protein kinase (DNA-PK)

Rolling the Hairpin

Viral DNA replication occurs in distinct stages. In the initial stage, following complementary strand synthesis, duplex RF DNA is amplified. In the next stage, individual single-stranded genomes are excised and displaced, initially feeding back as new templates into the replicative pool but later being consumed by encapsidation (5, 29, 31). During the amplification phase, a single continuous DNA strand, synthesized by a unidirectional fork driven by DNA polymerase δ , creates a series of concatemeric duplex intermediates (2, 31), as illustrated in **Figure 4** for the heterotelomeric negative-strand virus MVM. In step *i* the left telomere of incoming virion DNA folds back on itself, allowing the base-paired 3' nucleotide to prime synthesis of a complementary positive-sense strand. This creates a monomer-length duplex intermediate in which the two strands are joined at their left end via a single copy of the 3' hairpin, creating a turnaround form of the terminus. This duplex functions as a transcription template for NS1 expression, which is essential for all further steps in the replication pathway because the cellular machinery appears unable to melt and copy the viral 5' hairpin. The two hairpin telomeres play pivotal roles in the ensuing rolling mechanism, acting as hinges that first unfold, allowing themselves to be copied to generate an extended-form duplex copy of the entire hairpin (steps *ii* and *iii*). Because parvoviral hairpins are imperfect palindromes, the new flop sequence (*r*) can be recognized as the inverted complement of its flip template (*R*). This extended-form duplex is then melted and refolded into two hairpins (step *iv*), creating a rabbit-ear structure that pairs the 3' nucleotide of the newly synthesized DNA with an internal base, effectively reversing the path of the fork and redirecting it back along the internal coding sequences (step *v*). Both unfolding and refolding the hairpins require NS1, which binds site specifically to duplex motifs in the telomere and requires a functional SF3 helicase domain to assist in melting the duplex (100, 101). The fork then progresses back along the monomeric duplex, displacing the original negative strand and replacing it with a covalently continuous new strand. During fork progression, the turnaround form of the left hairpin is unfolded and copied, creating the dimer-junction sequence and leading first to the synthesis of a duplex dimer (step *vi*), which is then similarly processed (step *vii*) to a tetramer intermediate (step *viii*). Overall, the result of rolling hairpin synthesis is that the coding sequences of the virus are copied twice as often as the termini, and duplex dimeric and tetrameric concatemers are generated, in which alternating unit-length viral genomes are fused, through a single palindromic junction, in either a left-end:left-end or right-end:right-end configuration.

Turnaround termini: covalently continuous sequences joining two strands at the end of RF molecules

Extended-form termini: duplex copies of a terminal hairpin with NS1 covalently attached to their free 5' ends

Dimer-junction sequence: duplex copy of one entire left-end hairpin sequence that links two copies of the coding sequences in RF molecules

Resolving Hairpin Forms of the Telomere

Unit-length genomes are excised from these duplex intermediates, and their termini copied, by the introduction of a site- and strand-specific nick. When present as a turnaround structure at the

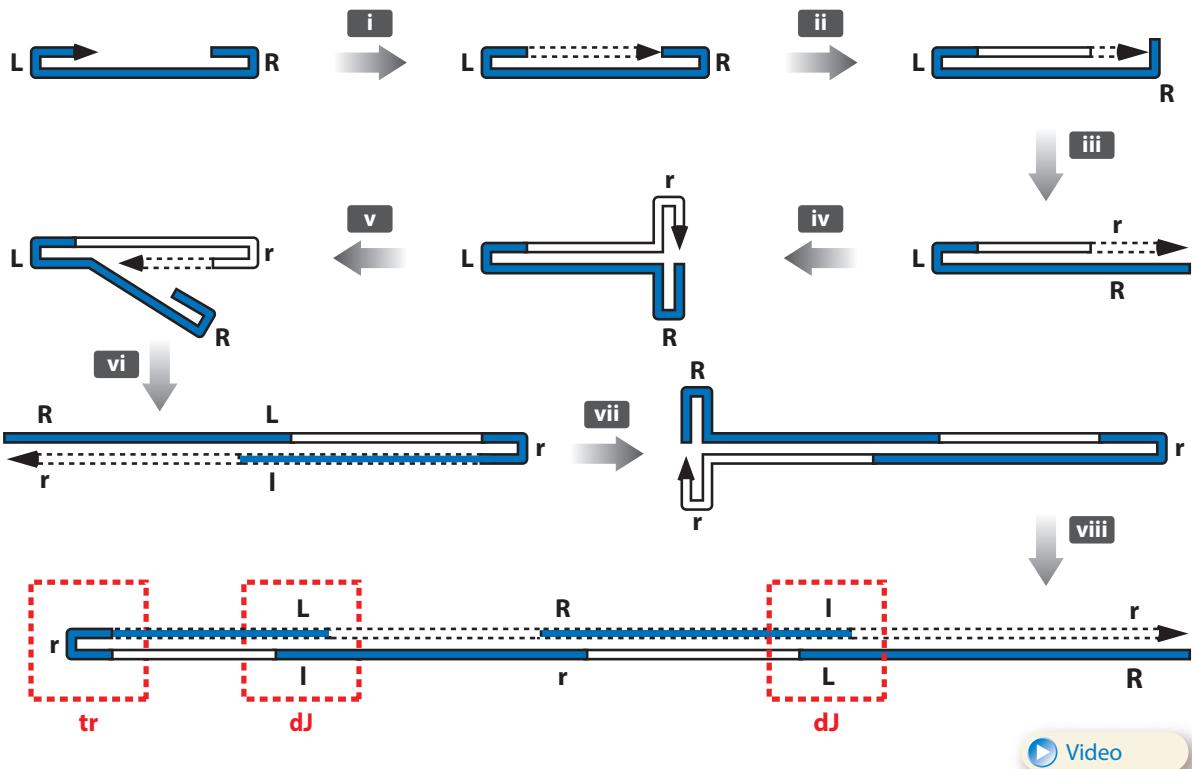


Figure 4

Schematic of steps in the rolling hairpin replication (RHR) model. L and l represent flip and flop forms of the left-end telomere; R and r represent these sequence arrangements of the right-end telomere. Newly synthesized DNA is represented by dashed lines, and its 3' end is capped with an arrowhead. Red dotted boxes designate the turnaround (tr) form of the right end and the dimer junction (dJ) form of the left-end palindrome. For an animation of the process, see [Video 1](#).

end of duplex RF (e.g., as shown in **Figure 4**, steps vi through viii), either of the AAV termini, but only the right-end hairpin of MVM, can be resolved by terminal resolution. As shown in detail in **Supplemental Figure 5a**, the AAV terminal palindromes are 125 nt in length and exhibit the lowest free energy when folded into T-shaped structures (2, 31, 102). Critical elements required for resolution of this structure include (a) a 22-base initiator-binding site (called RBE, for Rep-binding element), comprising five somewhat degenerate, tandem repeats of the initiator-binding motif, 5'-GAGC-3', which locates the nuclease on the DNA in the correct position and orientation for cleavage; (b) a nicking or terminal resolution site (*trs*), positioned in the upper strand 16 nt inboard of the RBE and flanked by short palindromic repeats that can rearrange into a stem-loop structure to present the *trs* as a single strand; and (c) an RBE' element, 5'-GTTTC-3', positioned at the tip of the hairpin arm opposite the nick site. Structural studies indicate that in the nicking complex, all three of these DNA elements make direct contact with the nuclease (32).

During terminal resolution, a single-strand nick is introduced into the DNA at the *trs*, via a trans-esterification reaction that transfers the phosphodiester bond to a tyrosine hydroxyl in the active site of the nuclease, leaving it covalently attached to the new 5' nucleotide and liberating a base-paired 3' hydroxyl to prime a new polymerase δ fork. This proceeds along the hairpin stem

toward its axis, first copying the lower strand and displacing the top strand, in a reaction that is equivalent to step *iii* in **Figure 4**, sequentially unfolding and copying the entire palindrome as its inverted complement. This inversion occurs every time a turnaround form of the telomere is resolved, ultimately generating an equimolar mix of flip and flop termini. Because both AAV termini can be nicked in the hairpin configuration, this equimolar mix occurs at both ends of the genome.

In heterotelomeric viruses such as MVM, however, left-end termini in the equivalent turnaround configuration cannot be nicked, and terminal resolution is confined to the right end of the genome. As seen in **Supplemental Figure 5b**, this much larger (248-nt) terminal hairpin can switch between a cruciform structure, as illustrated, in which a 36-bp palindrome containing reiterations of the NS1-binding motif, 5'-TGGT-3', folds into two arms, or a fully extended duplex form with just a few asymmetric nucleotides. ATP promotes the assembly of NS1 oligomers that bind asymmetrically over such motifs, protecting a 41–43-bp sequence that aligns with the 5' end of the (TGGT)₃ element but extends ~20 bp beyond its 3' end. The axial NS1 binding sites form an essential part of the nicking complex, even though they are positioned 120 bp from the cleavage site. NS1 also binds to a substantially weaker cluster of TGGT motifs that positions NS1 over the nick site. However, in order for nicking to occur, DNA-bending proteins from the HMGB family must coordinate interactions between the NS1 complexes bound at the two sites, creating an ~30-bp double-helical loop in the intervening G-rich DNA (103). Mutant hairpins that do not generate this loop in the presence of NS1 and HMGB do not nick, suggesting that it is critical for the activity of this hyperactive origin. After the nicking step, MVM right-end resolution resembles that of AAV, generating equimolar populations of flip and flop termini.

Resolving the Dimer Junction

As shown in **Supplemental Figure 4**, the stem of the MVM left-end hairpin contains an asymmetric bubble sequence, which prevents assembly of an active nicking complex on this structure. However, when the hairpin is unfolded and copied to form the dimer junction (**Supplemental Figure 6**), it can be resolved and replicated asymmetrically into two viral telomeres (104). Formation of the dimer junction effectively segregates two potential origins of DNA replication, one derived from each of the hairpin arms, on either side of the symmetry axis; however, only one of these is active, and its activity is again controlled by the sequence of the bubble. This serves as a precise spacer between the NS1 binding site and the binding site for the essential parvovirus initiation factor (PIF) (**Supplemental Figure 4**), binding of which stabilizes the interaction of NS1 with the active (TC) origin but not with the inactive (GAA) origin (105).

Junction resolution involves asymmetric nicking, synthesis of a heterocruciform intermediate, and the melting and reorientation of both arms of the duplex (30). Ultimately, it generates two new telomeres: One is derived from the inboard, GAA arm of the hairpin in the extended configuration, and the second, derived from the outboard, TC arm, is resolved into a turnaround configuration, as indicated in **Supplemental Figure 6**. This somewhat arcane process serves to maintain a single, flip sequence orientation at the left end of all full-length genomes. However, a mutant virus in which the hairpin ears were changed to the flop sequence maintained this sequence arrangement and was only marginally impaired, indicating that preservation of the flip orientation in the hairpin ears is a consequence of the asymmetric nature of dimer junction resolution but not the reason for it (106), which remains to be determined. One effect of employing this more constrained mechanism is that excision of the left-end termini is substantially slower than excision of their right-end counterparts, which moderates the release of 5-kb progeny genomes, instead directing displaced negative-sense strands that do not have a cleaved 3' terminus back into the RF

HMGB proteins:
chromatin
architectural
components that bind
into the minor groove
and distort the DNA
helix, promoting
site-specific binding by
many cellular factors

Bubble asymmetry:
spacing element
created by the
apposition of unpaired
bases, which disrupts
the duplex hairpin
stem

**Parvovirus initiation
factor (PIF):** a
heterodimeric cellular
transcription factor
also known as
glucocorticoid
modulatory
element-binding
protein (GMEB)

pool. As discussed below, this prevents rapid collapse of the amplification mechanism during the packaging phase of replication, allowing the sustained synthesis and release of progeny over many hours. Indeed, one possible explanation for the replicative asymmetry seen in MVM is that it is an evolutionary adaptation of an ancestral homotelomeric pattern, which has been selected because it ensures the sustained amplification of viral DNA throughout a protracted virion-release phase, which continues until the resources of the cell are exhausted and ultimately leads to the production of an enhanced virus load.

Displacing Single Strands, Packaging, and Releasing Progeny Virions

Single-stranded progeny genomes accumulate only during ongoing DNA replication and in the presence of preassembled empty capsids, suggesting that strands are packaged soon after, or concomitant with, release from the duplex template. Kinetic studies suggest that it is the increasing availability of capsids that directs the transition to this phase of replication (29). Because both termini of homotelomeric viruses are nicked and displaced via the same mechanism, with the same efficiency, the positive- and negative-sense strands displaced from these termini accumulate, and are packaged, with similar kinetics. However, heterotelomeric termini are processed quite differently, and the efficiency of nicking and rabbit-ear rearrangements at the right end of MVM exceeds that of left-end junction resolution, resulting in asymmetric progeny strand displacement and the preferential accumulation and packaging of negative-sense strands. Current packaging models largely derive from studies with LuIII, a heterotelomeric virus, which is atypical because it packages equal numbers of positive- and negative-sense strands, a property that maps to a 2-base insertion within the right-end origin, which effectively slows initiation. However, when these two nucleotides are removed, nicking efficiency recovers, and the resulting virus encapsidates predominantly negative-sense progeny. These observations show that encapsidation of a particular 3' terminus is not driven by a specific packaging signal.

Studies with AAV2 indicate that packaging is driven by the 3'-to-5' helicase activity of an independently expressed C-terminal domain of the initiator protein, called Rep^{40/52}. This serves as a capsid-tethered molecular motor that uses ATP hydrolysis to drive strand translocation into the capsid in a 3'-to-5' direction (9). The fate of newly released progeny strands is likely determined as their cleaved 3' ends are displaced from the template, because these must be captured by the packaging mechanism or else they will fold to prime additional duplex replication. NS1 can serve as the replicative helicase in reconstituted *in vitro* MVM systems, although whether the covalently attached molecule plays this role remains uncertain. In AAV, components of the cellular minichromosome maintenance (MCM) complex have also been implicated as the replicative helicase (107). Regardless of its other role(s), if the covalent 5' NS1 of the strand undergoing displacement is present in the replication fork when the 3' end is displaced, as suggested in **Supplemental Figure 7**, it would be available to mediate interactions with the capsid:helicase complex, effectively directing the packaging of the viral strand. Such an arrangement would allow LuIII to package strands of both senses despite the considerable differences between its two 3' termini. During packaging, DNA is pumped into the capsid through a fivefold pore, ultimately leaving its 5' sequences threaded through the packaging portal, connected via an exposed ~24-nt tether sequence to its 5'-associated NS1 molecule at the virion surface (2, 31). Because virions are released in this form, it is difficult to envisage how the 3' end of the DNA could remain in the packaging portal as the ensuing 5 kb follows it into the small virion. This topological problem suggests the two-portal model of virion packaging and uncoating, diagrammed in **Supplemental Figure 7**, which also takes into account the exceptionally dense nature of the full virion. For example, in virions from the genus *Protoparvovirus* approximately 33% of the DNA is also visible in the X-ray structure

and interacts through its bases with the inner capsid wall to establish noncovalent interactions with specific amino acid side chains (71, 108). Particle rigidity at the twofold axes of MVM derives predominantly from these capsid:DNA interactions (109, 110), perhaps indicating the importance of a full-length, 5-kb genome in establishing wild-type capsid dynamics, as also suggested by the *in vitro* uncoating studies (85).

Although the relative timing of virion release and lytic cell death has only been studied in detail for a few parvoviruses, MVM and HBoV1 virions packaged at early times in infection are known to be rapidly exported from the nucleus and released from viable cells, allowing infection to spread rapidly (80, 86, 111). In productively infected cells, the cell cycle is blocked prior to metaphase, by a variety of mechanisms (93, 94), creating a protracted pseudo-S phase that allows cells to survive for prolonged periods prior to lysis. Nuclear export of MVM virions is driven by their interaction with NS2 and the nuclear export protein CRM1 (49) and involves internalization in COPII vesicles in the endoplasmic reticulum and transport through the Golgi to the plasma membrane (12). This vesicular transport is gelsolin dependent and involves the rearrangement and destruction of actin filaments (112, 113). Within the Golgi, capsids are modified by tyrosine phosphorylation, and perhaps by other mechanisms, and the resulting virions show substantially improved particle-to-infectivity ratios compared with virus retrieved from intact cells (12).

Similar early export mechanisms have not been reported for AAV or B19, and the structures and dynamics of their fivefold cylinders do not suggest they could support an MVM-like system. Whether or not this strategy is beneficial only if linked to an asymmetric DNA replication mechanism that preserves and amplifies replicative-form DNA even after packaging initiates, and whether such asymmetry fulfills other roles in the viral life cycle, remains to be determined. Notwithstanding, these differences serve to highlight the enormous complexity and diversity of the pathways that have evolved in different lineages of the Parvoviridae despite the severely constrained genetic capacity of these minute viruses.

SUMMARY POINTS

1. Parvoviruses are small, rugged, nonenveloped protein particles containing a linear, non-permuted, single-stranded DNA genome with limited coding potential.
2. Typically, individual parvovirus strains are capable of infecting cells of a limited repertoire of differentiated phenotypes and require their hosts to be transiting the cell cycle of their own volition.
3. Viruses from this family use similar basic mechanisms to achieve specific steps in infection, but these processes have often evolved in directions that differ between genera.
4. Capsids have cylindrical surface projections surrounding each fivefold axis that function as dynamic portals, allowing helicase-mediated packaging of the genome into the pre-assembled particle and ultimately facilitating its vectorial ejection from the intact capsid.
5. In specific genera the cylinders also permit phased exposure of various VP N termini, which mediate specific steps in the cell entry process, including the coordinated exposure of a capsid-tethered phospholipase A2 domain present in VP1 N termini.
6. Upon entry into S phase, the genomic 3' hairpin telomere primes cellular replication machinery to synthesize a complementary viral strand, creating the first viral transcription template, the function of which depends upon its having intact left-end hairpin ears.

7. Parvoviruses employ rolling hairpin replication for rapid amplification of their DNA, synthesizing a series of linear duplex intermediates by a continuous single-strand displacement mechanism, mediated by the sequential folding and unfolding of the viral hairpin telomeres.
8. A subset of parvoviruses employ a rapid progeny virion export pathway that ensures early and continued progeny dissemination while the host cell continues to amplify viral DNA intermediates over an extended period prior to lytic cell death.

FUTURE ISSUES

1. Further insight into the mechanism(s) used by parvoviruses to take over the DNA replication program of their host cell is needed.
2. Stages in viral entry between endosomal escape and localization in the nucleus remain poorly understood.
3. Studies are required to determine the nature of the viral genome during cryptic infection and the trigger for its release, conversion to duplex DNA, and transcription early in the cellular S phase.
4. Genetic alterations occurring during neoplastic transformation that underlie the onco-selectivity of some rodent protoparvoviruses in human cells need to be identified.
5. How viruses evade the consequences of host sensors of virus replication, especially DNA damage response pathways, remains to be further elucidated.
6. Evidence for the continuous, nonlytic release of mature virions and what advantage(s) this confers on the virus needs to be assessed for members of each viral genus.

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