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PROBLEMS & PARADIGMS

Prospects & Overviews

Sequestering the 5'-cap for viral RNA packaging

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Abstract

Many viruses evolved mechanisms for capping the 5'-ends of their plus-strand RNAs as a means of hijacking the eukaryotic messenger RNA (mRNA) splicing/translation machinery. Although capping is critical for replication, the RNAs of these viruses have other essential functions including their requirement to be packaged as either genomes or pre-genomes into progeny viruses. Recent studies indicate that human immunodeficiency virus type-1 (HIV-1) RNAs are segregated between splicing/translation and packaging functions by a mechanism that involves structural sequestration of the 5'-cap. Here, we examined studies reported for other viruses and retrotransposons that require both selective packaging of their RNAs and 5'-RNA capping for host-mediated translation. Our findings suggest that viruses and retrotransposons have evolved multiple mechanisms to control 5'-cap accessibility, consistent with the hypothesis that removal or sequestration of the 5' cap enables packageable RNAs to avoid capture by the cellular RNA processing and translation machinery.

KEYWORDS

5'-cap, cap sequestration, RNA packaging, RNA virus, translation regulation

INTRODUCTION

Eukaryotic messenger RNA (mRNA) transcribed by the DNA-dependent RNA polymerase II (Pol II) are co-transcriptionally capped at their 5' ends by a 5'-triphosphate linked 7-methylguanosine residue (⁷mGpppN-, also known as the 5'-cap). Cap-synthesis is carried out by Pol II-associated capping enzymes and occurs early during transcription, likely after the incorporation of 10–20 nucleotides.^[1] The 5'-cap is an essential post-transcriptional modification that plays

critical roles in many RNA functionalities, including pre-mRNA splicing, 3'-polyadenylation, RNA nuclear export, resistance to 5'-3' exonucleases, and translation initiation.^[2] The functions of the 5'-cap are achieved by its interactions with various cap-binding proteins, mainly the cap-binding complex (CBC) in the nucleus and the eukaryotic translation initiation factor 4E (eIF4E) in the cytoplasm.^[3] The cap-CBC interactions facilitate mRNA maturation and nuclear export, while eIF4E binding to the mRNA 5'-cap initiates translation. The predominant mechanism of eukaryotic translation initiation is cap-dependent: the pioneer round of translation is mediated by the cap-associated CBC protein, which is then replaced by eIF4E for the following steady-state translation.^[2,4] In both cases, cap-associated CBC or eIF4E serves as the anchor site to recruit additional translation factors such as the eIF4G and RNA helicase eIF4A and eventually the 40S and 60S ribosomal subunits for successful translation initiation.^[4,5] The 5'-cap also protects RNAs from degradation by cellular exoribonucleases.^[6]

Abbreviations: CBC, cap-binding complex; 3'-CS, 3'-consensus sequence; dsRNA, double-stranded RNA; eIF4E, eukaryotic translation initiation factor 4E; gRNA, genomic RNA; GTase, guanylyltransferase; HBV, hepatitis B virus; HIV-1, human immunodeficiency virus type-1; IAV, influenza A virus; LTR, long terminal repeat; MTase, methyltransferase; NC, nucleocapsid; NSP, non-structural protein; NSV, negative-sense RNA virus; ORF, open-reading frame; pgRNA, pre-genomic RNA; Pol II, RNA polymerase II; preC-RNA, pre-core protein mRNA; RdRp, RNA-dependent RNA polymerase; RTPase, RNA triphosphatase; SAM, S-adenosyl methionine.

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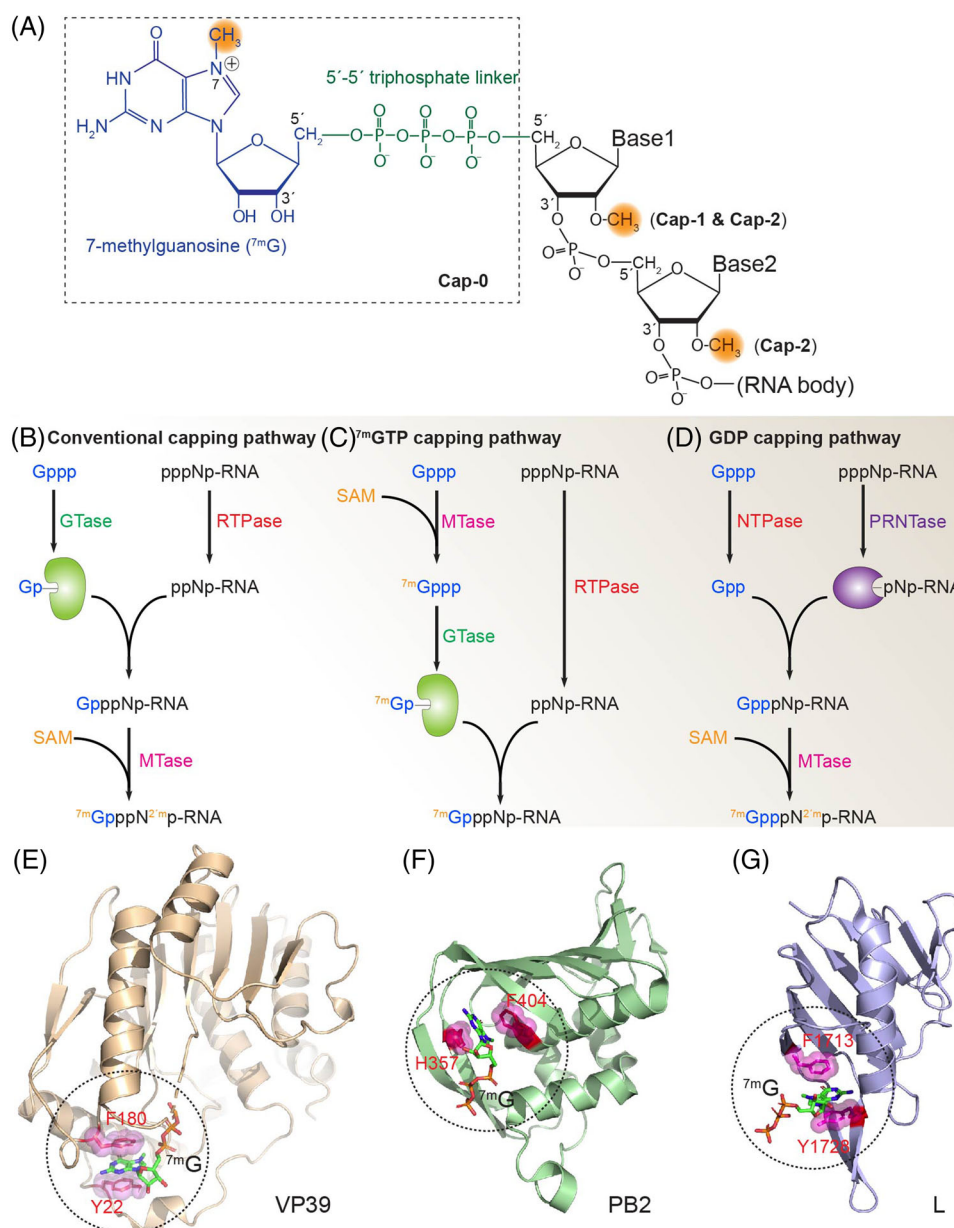


FIGURE 1 Viral RNA 5'-cap. (A) Structure of the 5'-cap of viral mRNA. A 7-methylguanosine (blue) is linked to the RNA 5'-end through a 5'-5' triphosphate linker (green). The methylations on the N7 and 2'-O are highlighted in orange. (B-D) Viral RNA capping mechanisms. All capping pathways start with GTP (Gppp) and the nascent RNA (pppNp-RNA). (E-G) Viral cap binding proteins and their cap-recognition mechanisms. In each protein, two residues with aromatic ring sandwich the 7-methylguanosine cap (7^mG) through cation- π interactions. Additional hydrogen bonds further stabilize the protein/cap complex (not shown in the figure). GTase, guanylyltransferase; L, Bunyavirus multifunctional polymerase (PDB ID 6QHG); MTase, methyltransferase; NTPase, nucleoside triphosphatase; PB2, influenza virus polymerase subunit (PDB ID 4C4B); PRNTase, polyribonucleotidyl transferase; RTPase, RNA triphosphatase; SAM, S-Adenosyl methionine; VP39, vaccinia virus MTase (PDB ID 1AV6)

Eukaryotic mRNAs are often methylated at the 2'-hydroxyl group (2'-O) of the first and second nucleotides following the 5'-cap (Figure 1A), and occasionally at the 2'-O positions of additional downstream nucleotides,^[7] and RNAs lacking a 5'-cap structure or the 2'-O methylation(s) can be detected by host restriction factors and trigger immune responses as a means of responding to non-self RNAs.^[8-10]

Viruses have evolved diverse mechanisms to co-opt the host translational machinery for viral protein production.^[11,12] While some viruses use a viral protein covalently linked to the 5'-end of viral mRNA (viral

protein genome-linked, VPg) and/or a *cis*-acting internal ribosomal entry site (IRES) RNA element (such as RNA viruses from the family of *Picornaviridae*),^[13,14] the major way for a viral RNA to hijack the cellular translation machinery is to mimic the host cellular mRNAs by exploiting the cap-dependent translation initiation mechanism.^[12] As discussed below, a diverse set of mechanisms have been utilized by different viruses to synthesize the cap structure at the 5'-end of their plus-strand RNAs. In addition to functioning as mRNA for the translation of structural and/or functional proteins, the viral plus-strand RNAs of all

RNA viruses and some DNA viruses perform other essential functions during the replication. The plus-strand RNA of retroviruses (single-stranded positive-sense RNA virus) is selectively incorporated into assembling virions as the progeny genome (genomic RNA [gRNA]).^[15] Plus-strand RNAs are also specifically packaged as pre-genomes in rotaviruses (double-stranded RNA [dsRNA] virus)^[16] and hepatitis B virus (HBV, double-stranded DNA virus).^[17] In some RNA viruses, the plus-strand RNA is also critical for replicating the viral RNAs by serving as the template for the generation of the minus-strand RNA, such as in influenza viruses (single-stranded negative-sense RNA virus [NSV])^[18] and flaviviruses (single-stranded positive-sense RNA virus).^[19] Thus, the plus-strand RNAs of diverse viruses are essential for both translational and non-translational (replication and/or packaging of the viral genomes) functions.

A major question is how processing/translation versus packaging functions of the plus-strand RNAs are regulated.^[20] Influenza viruses produce two different types of plus-strand RNAs: a capped form that functions as mRNA and a noncapped form functions as the template for RNA replication.^[18,21] Some retroviruses also have two distinct pools of full-length plus-strand RNA, one that is retained in the cell for translation (mRNA) and the other that is selected for packaging (gRNA).^[22,23] Recent studies in human immunodeficiency virus type-1 (HIV-1) revealed that the mRNA versus gRNA functions of the plus-strand RNA is governed by the accessibility of the 5'-cap, with cap-sequestration being an essential determinant for efficient packaging.^[24,25] Here, we summarize the role of the 5'-cap in regulating the function of viral plus-strand RNAs and emphasize its role in viral RNA packaging. We hypothesize that control of cap accessibility by de-capping or cap sequestration could be a general mechanism for expansion and control of plus-strand RNA function.

VIRUSES USE DIVERSE MECHANISMS TO CAP THEIR PLUS-STRAND RNAs

Most viral mRNA 5'-caps are indistinguishable from that of the cellular mRNA: a 7-methylguanosine moiety appended to the viral RNA 5'-end through a triphosphate linker (^{7m}GpppN-, cap-0) (Figure 1A). For a small number of viruses, the 5'-cap is hypermethylated with additional modification(s) at the N2 position of the cap residue^[26–30] which is a hallmark of the cellular small nuclear, small nucleolar and telomerase RNAs.^[31,32] The 2,2,7-trimethyl guanosine (TMG) cap of a subset of HIV-1 mRNAs was shown to initiate translation in a CBC-dependent manner rather than requiring eIF4E binding.^[33,34] The extra methyl group(s) in the hypermethylated cap is added by a unique methyltransferase (MTase).^[32,33]

Viruses have evolved multiple mechanisms for capping their RNAs.^[11,35] The mRNAs of most nucleus-replicating DNA viruses (HBV) and retroviruses (HIV-1) are synthesized by the host Pol II and capped by the normal cellular capping apparatus. For the capping of eukaryotic mRNAs (also called the conventional cap-synthesis pathway, Figure 1B), the RNA triphosphatase (RTPase) activity removes the γ -phosphate from the terminus of the 5'-

triphosphorylated mRNA, then the guanylyltransferase (GTase) activity catalyzes the transfer of a guanine monophosphate (GMP) to the 5'-diphosphorylated mRNA (GpppNN-), which is followed by the transfer of a methyl group from S-adenosyl methionine (SAM) to the N7 position of the terminal guanine (^{7m}GpppNpN-) by (guanine-7)-methyltransferase (N7MTase) and the nucleoside 2'-O position of the following nucleotides (^{7m}GpppN^{2'm}pN- or ^{7m}GpppN^{2'm}pN^{2'm}) by (nucleoside 2'-O)-methyltransferase (2'OMTase). The collective catalytic reactions of RTPase, GTase, and MTases are referred to as the capping process, which are encoded either in separate polypeptides (e.g., in yeast) or as different domains of a single protein (such as the RTPase-GTase in metazoans).^[3,11]

Many viruses with a cytosolic replication cycle encode their own capping enzymes, some of which utilize the conventional capping mechanism of eukaryotic cells. The rotavirus VP3 multidomain protein contains all the RTPase, GTase, N7MTase, and 2'OMTase activities,^[36] and the flavivirus NS3 protein has the RTPase activity while its RNA-dependent RNA polymerase (RdRp) NS5 also performs the GTase and MTase reactions (Figure 1B).^[37] Other viral capping enzymes have evolved unique "unconventional capping mechanisms" with cap synthesis pathways distinct from the eukaryotic capping mechanism. In alphaviruses, the N7MTase activity of the nsP1 replicase protein transfers a methyl group from SAM to the N7 position of GTP and then the GTPase activity of nsP1 appends the ^{7m}GMP to the 5'-end of the diphosphorylated RNA generated by the RTPase activity of nsP2 (Figure 1C).^[11] SARS-CoV-2 also employs an unconventional capping mechanism, in which its nsP12 protein transfers a guanine diphosphate moiety (GDP rather than a GMP) to the monophosphorylated viral RNA from the nsP9-p-RNA intermediate (also synthesized by nsP12), which is followed by the methylation by nsP14 (N7MTase) and nsP16 (2'OMTase) proteins.^[38] Capping processes similar to SARS-CoV-2 have also been discovered in *Rhabdoviridae*-like viruses (Figure 1D).^[11]

Some viruses do not encode their own capping enzymes but instead steal the 5'-cap from host mRNAs by a mechanism known as cap-snatching, which has been well-characterized in the single-stranded NSVs from the families of *Orthomyxoviridae* and *Bunyaviridae*.^[21,39] Cap-snatching is initiated by the binding of the cellular mRNA 5'-cap to the cap-binding site of a viral multi-functional protein, followed by the cleavage of the cellular mRNA at 1–20 nucleotides downstream of the 5'-cap. The resulting capped RNA fragment is then used to prime the transcription of the viral mRNA by its RdRp. Both the cap-binding and endonuclease activities are carried out by the viral polymerases in influenza virus (*Orthomyxoviridae*)^[21] and bunyaviruses,^[39] whereas the nucleocapsid (NC) protein of Hantavirus is proposed to have the corresponding cap-binding site.^[40] Cap-snatching enables efficient translation of viral mRNAs, while the de-capped host mRNAs are targeted for degradation.

Although the viral and cellular cap-binding proteins have no sequence homology and low overall structural similarity, most of them have similar cap-recognition mechanisms: two aromatic rings sandwich the cap moiety with cation- π stacking interactions, with binding further stabilized by specific intermolecular hydrogen bonds (Figure 1E, F). Although the Hantavirus N protein contains the potential cap

binding site for cap-snatching,^[40] no classical cap-binding pocket with aromatic residues has been identified in its three-dimensional structure,^[41] suggesting a different cap-recognition mechanism. In addition, a distinct cap-binding mode has been reported for the cap-binding site of rotavirus VP1 protein (see below).^[42] The binding of cellular proteins to the 5'-cap plays known roles in regulating viral translation, replication, and packaging, could conceivably also play an inhibitory role in the innate immune response.^[43,44]

CAP SEQUESTRATION IS REQUIRED FOR HIV-1 RNA PACKAGING

HIV-1 is a retrovirus with single-stranded positive-sense RNA genome. Upon entering cells, the gRNA is copied into double-strand DNA by the viral reverse transcriptase and integrated into the host chromosome, conferring life-long infection.^[45] The integrated proviral DNA is subsequently transcribed by the host Pol II and the RNAs co-transcriptionally capped using the cell's mRNA-capping machinery as discussed above.^[46] A fraction of the capped full-length HIV-1 RNA transcripts function as mRNAs that are translated into the viral Gag and Gag-Pol proteins, the protein building blocks of the virus. Gag-Pol contains peptide regions including the viral protease, reverse transcriptase, and integrase, which are proteolytically liberated during viral maturation.^[47] Full-length HIV-1 RNAs also serve as the genome for progeny viruses, and HIV-1 mRNA and gRNA functions were originally proposed to be conferred by a single RNA species.^[48–50] However, early studies of murine leukemia virus^[51] and more recent studies of HIV-1^[52–54] suggest a mechanism in which the mRNAs and gRNAs exist in distinct, non-interconverting pools.

Like nearly all other retroviruses, HIV-1 specifically and efficiently packages two copies of its gRNA in the form of non-covalently linked dimer into the assembling virion, which is selected from a cellular milieu that contains a substantial excess of non-viral RNAs and viral spliced and unspliced mRNAs.^[55–57] gRNA packaging by HIV-1 is mediated by specific interactions between the NC domain of the viral Gag protein and the *cis*-acting RNA “packaging signal” located within an ~350 nt region at the 5'-end of the 9-kilo-bases (kb) genome (also known as the 5'-leader).^[58–61] The 5'-leader is the most conserved region of the HIV-1 genome and contains multiple functionally important RNA elements. Studies indicate that the functions of the 5'-leader are modulated by its dimerization state, and that the monomeric and dimeric forms of the 5'-leader adopt distinct secondary and tertiary structures.^[24,57,60,62–79] Over the past three decades, more than 20 secondary structures have been proposed for the 5'-leader on the basis of mutagenesis experiments, free energy calculations, chemical probing, and NMR, and although there is consensus that transcriptional activation, primer binding, dimerization, and splicing activities are promoted by discrete hairpin structures (TAR, PBS, DIS, and SD hairpins, respectively),^[24,57,60,62–79] there has been little consensus regarding other structural features. This is likely due in part to the fact that the 5'-leader adopts multiple structures both *in vitro* and in cells, which can confound analysis of chemical probing data.^[79] Indeed, results of in-gel

chemical probing of the dimeric 5'-leader^[80] are more compatible with the NMR-derived structure than with models proposed on the basis of chemical probing and free energy predictions.^[79] Additionally, it has recently been shown that a widely used method for probing nucleotide accessibility (selective 2'-hydroxyl acylation analyzed by primer extension, SHAPE)^[81] does not robustly identify conformationally dynamic nucleotides.^[82] SHAPE is potentially attractive because it enables structural probing of large RNAs not only *in vitro*, but also in living cells and viruses. However, the approach assumes that the probability of a chemical modification is reflected by the extent of termination or mutation that occurs at a modified site during reverse transcription (RT)-based readout, and it is now known that some modified bases are largely invisible to readout by RT termination or mutational mapping alone,^[83] and that RT mutations and stops are poorly correlated, are subject to different biases, and represent largely discrete sets of information from chemical probing of RNA structure.^[84] The use of multiple methods for detecting sites of nucleotide modification is likely to improve the reliability of RNA secondary structure predictions derived from chemical probing experiments.^[84] The secondary structures outlined in Figure 2 are based on NMR methodologies^[85] and the dimer structure appears consistent with a recent structural analysis by small angle X-ray scattering.^[86]

Although 5'-leader dimerization was initially thought to be regulated by a riboswitch-like mechanism of a single viral RNA transcript, recent studies indicate that the dimerization state of HIV-1 transcripts is controlled at the level of transcription by a heterogeneous transcriptional start site mechanism.^[24,53,54] HIV-1 proviral DNA only contains one promoter, but heterogeneous start site usage generates functionally distinct RNA pools that differ by the inclusion of one or two 5'-guanosines.^[24,53,54] RNAs beginning with a single 5'-guanosine (1G) adopt the dimeric conformation that exposes high affinity NC binding sites and thus promotes its packaging as gRNA (Figure 2A). In contrast, HIV-1 RNAs that begin with two or three 5'-guanosines (2G or 3G, respectively; the dominant form is 3G) adopt a monomeric conformation with fewer NC binding sites and are retained in cells as mRNAs^[24,54,78] (Figure 2B). Although both the 1 and 2G/3G forms of the RNA transcripts are capped co-transcriptionally, *in vitro* NMR experiments showed that the 5'-cap in the 1G form of the 5'-leader (^{Cap}1G-L) is buried in a co-axial stacking structure of the TAR and PolyA hairpins and is thus inaccessible for the cap-binding eukaryotic translation initiation factors (Figure 2A). However, the 5'-cap in the 2G/3G form of the 5'-leader (^{Cap}2G-L or ^{Cap}3G-L) is exposed and readily binds the cap-binding protein eIF4E (Figure 2B).^[24] It was recently shown that a fraction of HIV-1 mRNAs are hypermethylated, which appears to enable translation initiation using the nuclear cap-binding protein rather than eIF4E.^[33] It remains to be determined if cap hypermethylation is dependent on 5'-guanosine number.

The RNA determinants in the dimeric 5'-leader responsible for genome packaging have been extensively studied.^[25,60,61,65,87–91] All of the high-affinity NC binding sites (ca. two dozen) are located in an ~150-nt region of the leader, which is the minimum region required for leader dimerization and is sufficient to promote packaging of heterologous RNA into HIV-1 virus-like particles.^[60,61] This

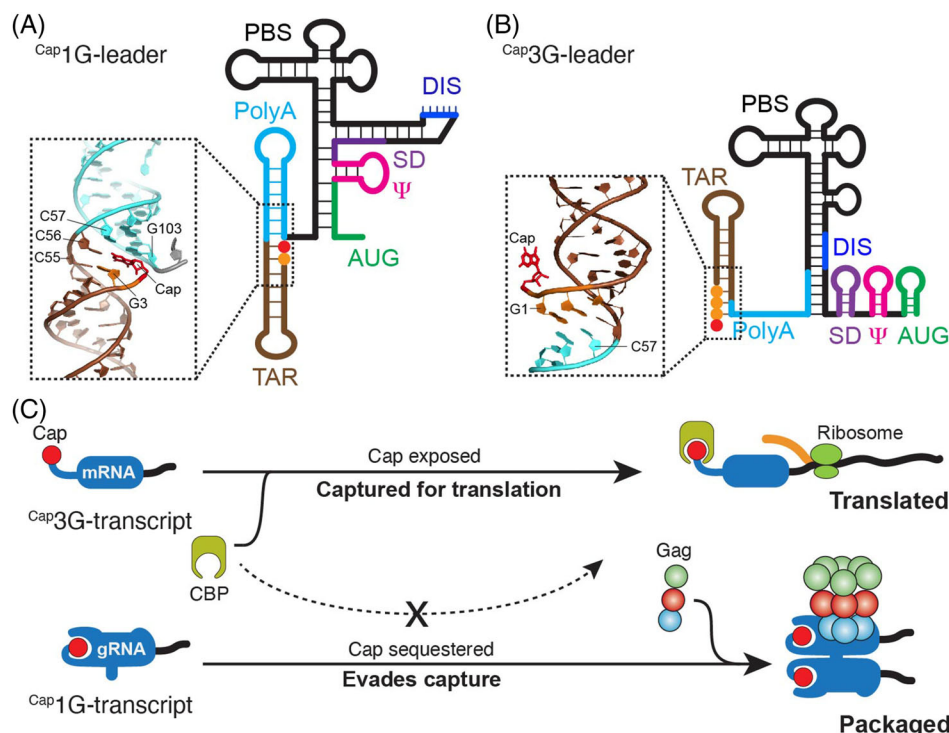


FIGURE 2 Cap-sequestration promotes HIV-1 gRNA packaging. (A) Cartoon representation of the leader region of HIV-1 RNA transcript that starts with a single 5'-guanine, derived by NMR (orange sphere, 1G-leader).^[24,79] The 5'-cap (red sphere) is sequestered by the co-axial stacking of the TAR and PolyA helices (insert, PDB ID 6VVJ). The ^{Cap}1G-leader is dimerization competent with the DIS exposed and promotes packaging. (B) NMR-derived structural model of the leader region of HIV-1 RNA transcript with three 5'-guanines (3G-leader).^[24] The 5'-cap is exposed (insert, PDB ID 6VU1) and accessible to cellular cap-binding proteins. The ^{Cap}3G-leader adopts a monomeric conformation with the DIS region sequestered by intramolecular base-pairing. (C) Proposed working mechanism for HIV-1 selective gRNA packaging. The ^{Cap}3G-transcript exposes the 5'-cap which is recognized by the cellular CBP for translation and thus retains in the cell and functions as mRNA. The ^{Cap}1G-transcript sequesters the 5'-cap to evade the capture by cellular translation machinery and is selected for packaging by viral Gag proteins. Adapted from ref.^[25] which is licensed under CC BY-NC-ND. CBP, cap-binding protein; DIS, dimerization initiation site; gRNA, genomic RNA; HIV-1, human immunodeficiency virus type-1

"core encapsidation signal" or Ψ^{CES} , which lacks that the TAR-PolyA helices and the PBS regions, adopts a tandem three-way junction structure.^[61,79] Despite containing all the high-affinity NC binding sites, Ψ^{CES} is poorly packaged when assayed in competition with the wild-type 5'-leader. The additional determinants required for wild-type packaging were mapped to the TAR-PolyA tandem hairpins that sequester the 5'-cap.^[24,25,92] Mutations in the full-length leader that expose the cap without affecting its dimerization or NC binding properties abrogate packaging, and addition of a cap-shedding ribozyme to Ψ^{CES} restores packaging efficiency to that of the native 5'-leader.^[25] These findings collectively demonstrate that, in addition to NC-recognition, cap-sequestration is an essential determinant of HIV-1 RNA packaging. Since 5'-cap is critical for RNA splicing and translation, cap sequestration appears to function by preventing dominant negative cap-dependent capture by the cellular RNA processing and translation machinery (Figure 2C). Interestingly, cellular non-coding RNAs lack a 7-methylguanosine cap are also enriched in retroviruses,^[93–95] consistent with the hypothesis that cap sequestration is required for RNA packaging. More recent studies confirmed that transcriptional start site heterogeneity and preferential packaging of specific 5'-capped full-length RNA species are conserved proper-

ties among the primate lentiviruses.^[96] Further studies are warranted to characterize the cap accessibility and its relationship to RNA fate determination in these viral RNAs. For retroviruses that do not exhibit transcriptional start site heterogeneity, we speculate that the originally proposed riboswitch-like mechanism that influences exposure of viral Gag binding sites^[97] might also modulate cap accessibility (a hypothesis that has not yet been tested). A very recent study suggested that N⁶-methyladenosine deposition could play a role in regulating transcription versus packaging functions of individual HIV-1 RNA transcripts.^[98] Although this mechanism is at odds with evidence that translation and packaging are mediated by different transcripts, the proposed role for epitranscriptomic regulation of HIV-1 RNA function is intriguing and deserves further exploration.

RETROTRANSPOSONS PACKAGE DE-CAPPED/UNCAPPED RNAs INTO VIRUS-LIKE PARTICLES

Cap-dependent modulation of RNA packaging has also been reported in long terminal repeat (LTR) retrotransposons, which are transposable DNA elements that replicate through RNA intermediates and are

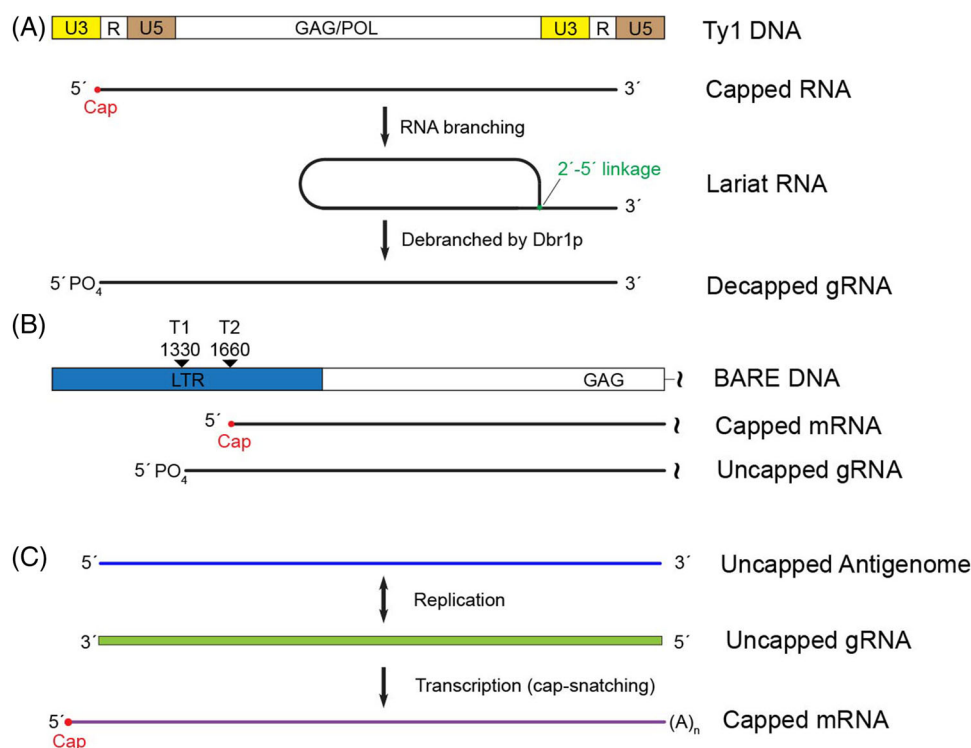


FIGURE 3 Packaging of noncapped RNAs as the viral genomes. (A) Structural organization of the genomic DNA and RNA transcript of the yeast Ty1 retrotransposon. R is the region repeated at the 5' and 3' end of the RNA transcript, U5 and U3 are the unique regions to the 5'- and 3'-end, respectively. Some of the Ty1 capped RNA transcript undergoes a branching process in which the RNA 5'-end is covalently linked to a 3'-proximal nucleotide through a 2'-5' linkage. Debranching by the RNA lariat debranching enzyme Dbr1p generates RNA with 5'-monophosphate, which is accumulated in Ty1 VLPs. (B) The retrotransposon *BARE* uses two distinct RNA pools for translation and packaging. The RNA transcript from promoter 2 (T2) is 5'-capped and 3'-polyadenylated to serve as the mRNA, while the RNA transcript from promoter 1 (T1) lacks 5'-cap and 3'-polyadenylation which is packaged into the VLP as genomic RNA (Grna). (C) Negative-strand RNA virus produces two distinct plus-strand RNAs, one is capped through cap-snatching and functions as mRNA and the other is not capped and serve as the template for transcription and replication. The negative-strand RNA genome is also not capped

ubiquitously found in many eukaryotic genomes.^[99] LTR retrotransposons are considered as the evolutionary progenitors of retroviruses. They replicate through a “copy-and-paste” mechanism in which their RNA transcripts are reverse transcribed back into cDNAs that are then inserted into new genomic loci.^[100,101] The Ty1 element of *Saccharomyces cerevisiae* is among the best characterized LTR retrotransposons. Ty1 is 5.9 kb in length and contains two ~300 nucleotide terminal repeats with the sequences in between coding for the structural Gag protein and enzymes required for transposition (Figure 3A). The virus-like particle assembled from Gag proteins selectively packages the Ty1 RNA transcript as the template for RT. Ty1 RNA is transcribed by the host Pol II and is thus co-transcriptionally capped. Although most of the Ty1 RNAs in the cell are capped, the major form of RNA packaged into the virus like particle is uncapped, which is due to RNA branching and debranching events.^[102] The 5'-cap of some Ty1 RNAs is removed by joining the 5'-end and a nucleotide close to the 3'-terminus through a 2'-5' phosphodiester bond (Figure 3A). Breaking down of the 2'-5' bond by the host RNA lariat debranching enzyme Dbr1 generates the linear RNA with a single phosphate group at the 5'-end, which is the RNA species enriched in the virus

like particle.^[102,103] While the branching process is required for 5'-cap removal, the debranching event has been suggested to play important roles in RT.^[103,104] Thus, Ty1 RNA transcripts with 7-methylguanosine cap function as mRNA while the de-capped RNA transcripts containing a monophosphate 5'-end are likely selectively incorporated into the virus like particle as the template for RT.

Selective packaging of uncapped RNA is also utilized by the *BARE* LTR retrotransposon, another Ty1/Copia superfamily member constituting over 10% of the barley genome. The *BARE* LTR region contains two sequential promoters separated by ~300 nt, which produce two groups of RNA transcripts with different lengths (Figure 3B).^[105] RNAs generated from these two promoters share most of their sequences except that the longer transcripts from the first promoter (TATA-1) lack 5'-cap and 3'-polyadenylation modifications (Figure 3B). It was found that the uncapped TATA-1 transcripts are selectively packaged into virus-like particles while the capped TATA-2 transcripts are spliced and/or translated.^[106] Thus, *BARE* LTR retrotransposon selectively packages its uncapped RNA transcripts, which are produced directly by transcription from a unique promoter. Generation and packaging of uncapped gRNAs were also discovered in RNA

viruses including the single-stranded positive-sense RNA containing alphaviruses.^[107,108] Significant amounts of uncapped gRNAs are produced early during infection, which has been shown to be critical for alphavirus pathogenicity.^[108] It is thus conceivable that uncapped gRNAs increase the packaging efficiency by alphaviruses as well as other plus-strand RNA viruses.

NEGATIVE-SENSE RNA VIRUSES PACKAGE UNCAPPED RNAs

Uncapped gRNAs are also packaged into NSVs such as influenza A virus (IAV) and Bunyaviruses. In these cases, the RNA genomes are antisense to their mRNAs and are thus not capped for translation.^[18] After cells are infected, the NSV RdRp performs both replication and transcription using the same RNA template to generate two types of plus-strand RNAs with distinct functions. Replication by RdRp produces the plus-strand antigenome, which is the complementary copy of the negative-strand RNA genome.^[18] The antigenome is further used as the template by RdRp to generate more negative-strand gRNAs that are packaged into the progeny viruses. Both the negative-strand gRNA and the plus-strand antigenome are uncapped. On the other hand, the plus-strand transcription products of the gRNA by RdRp are capped either by virally encoded capping enzyme (non-segmented negative-strand RNA viruses) or a cap-snatching mechanism as mentioned above (segmented negative-strand RNA viruses)^[21,39] and these capped plus-strand RNAs function as mRNA to be translated into viral proteins. Thus, the cap status of these plus-strand RNAs plays a critical role in modulating the RNA translation and replication/packaging function of the NSV plus-strand RNAs (Figure 3C).

PROTEIN BINDING AS A POTENTIAL MECHANISM FOR CAP SEQUESTRATION AND VIRAL RNA PACKAGING

The genome of rotaviruses is comprised of 11 segmented dsRNA fragments with their lengths ranging from 0.7 to 3.1 kb. During rotavirus assembly, each of the 11 genome fragments is selectively incorporated in its 5'-capped and non-polyadenylated plus-strand form.^[16] In addition to functioning as the template for genome replication, each of the plus-strand RNAs generally contains a single open-reading frame (ORF) flanked by untranslated regions, and these RNAs function as the mRNA for one of the structural proteins (VPs) or non-structural proteins (NSPs) of the virus. Rotavirus plus-strand RNAs likely contain sequence and structure elements that promote their packaging. Computational studies and RNase mapping experiments suggest that 5'-3' base-pairing of the plus-strand RNA termini creates a panhandle structure with a highly conserved single-stranded 3'-terminus ("UGUGACC" for group A rotavirus, Figure 4A). This 3'-consensus sequence (3'-CS) is directly recognized by the viral RdRp (VP1), which is believed to facilitate the packaging of plus-strand RNAs into the viral particle.^[109,110] The core shell of the viral particle is composed of viral VP2 protein and

engagement of VP2 activates the enzymatic activity of VP1 to initiate the synthesis of the minus-strand RNA and the production of the dsRNA genome within the virial core particle.^[16] VP1 is associated with the viral capping enzyme (VP3), which adds the 7-methyl guanosine cap to the plus-strand RNA in the next round of replication.^[36] Importantly, a cap-binding site has been identified on the surface of the VP1 polymerase, and the VP1/5'-cap interaction in the panhandle structure of the plus-strand RNAs has been proposed to further stabilize the VP1/3'-CS interactions for RNA packaging.^[16,42] While the 5'-cap is associated with the viral VP1 polymerase when the plus-strand RNAs function as the replication templates for packaging, the 5'-cap is bound by eIF4E for translation initiation when the plus-strand RNAs function as mRNA, in which the 3'-CS is occupied by the viral NSP3 protein instead of VP1 (Figure 4A).^[111,112] NSP3 is thought to be a surrogate of the polyA binding protein, which promotes translation by enhancing 5'-3' communication of the mRNAs through its binding to both the 3'-polyA signal and the 5'-cap associated eukaryotic initiation factors eIF4G.^[113,114] Since packaging and translation are conflicting functions of the rotavirus plus-strand RNAs and the 5'-cap is critical for translation initiation, it seems plausible that sequestration of the 5'-cap by the viral VP1 polymerase may suppress the translation activity of the plus-strand RNAs, similar to the RNA structure-dependent cap sequestration observed for HIV-1.^[25]

Viral polymerase-mediated suppression of translation to promote plus-strand RNA packaging has been reported for HBV, the prototypic member of the *Hepadnaviridae*. HBV is a small enveloped virus that contains a partially double-stranded DNA genome (also called the relaxed circular DNA or rcDNA), which is repaired into a covalently closed circular DNA genome (cccDNA) in the nucleus of infected cells.^[17] Transcription by the host RNA Pol-II generates 5'-capped and 3'-polyadenylated viral RNAs, including the pre-gRNA (pgRNA) and other viral mRNAs. Concomitant with viral capsid assembly, one copy of the pgRNA is selectively incorporated and then reverse transcribed into the rcDNA genome by the virally encoded polymerase (HBV-Pol or P protein). Selective encapsidation of pgRNA is mediated by specific interactions between the HBV-Pol and a *cis*-acting encapsidation signal (called the ϵ -element) located at the 5'-end of the 5'-capped pgRNA (Figure 4B).^[115-117] Because of the terminal redundancy of pgRNA and the co-terminal nature of all viral transcripts, the ϵ -element is also present at the 3'-ends of all the sub-genomic viral mRNAs, which however does not direct selective RNA packaging.^[117] More intriguingly, the packaging deficient mRNA of pre-core protein (pre-core protein mRNA [preC-RNA]) only differs from pgRNA by an additional ~30-nt at its 5'-end, which is also full-length and contains all the sequences of pgRNA (Figure 4B). In vivo packaging experiments with RNA constructs containing the ϵ -element inserted at different locations revealed that the ϵ -element and its appropriate distance (~30 nt in length) to the 5'-cap are both critical for wild-type packaging efficiency.^[118] It was also shown that the binding of the HBV-Pol to the ϵ -element suppresses the translation of pgRNA, which is also dependent on the proximity between the 5'-cap and the ϵ -element in a similar manner.^[119,120] Therefore, translation suppression of pgRNA by HBV-Pol and its

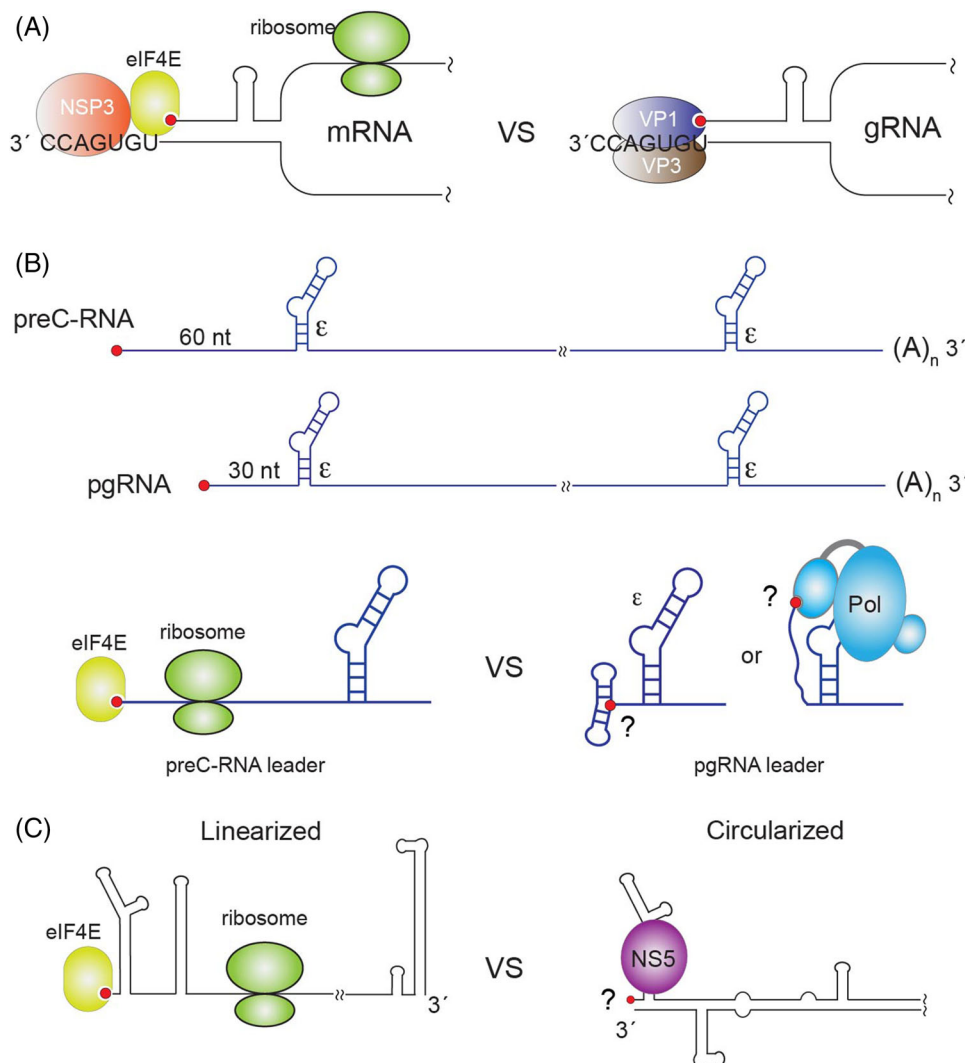


FIGURE 4 Potential protein-mediated cap-sequestration may contribute to gRNA packaging in other viruses. (A) The rotavirus plus-strand RNA forms a panhandle structure due to base-pairing of the 5'- and 3'-ends. When functioning as mRNA, the 5'-cap and 3'-consensus sequence (UGUGACC) are bound by eIF4E and NSP3, respectively, which promotes efficient translation. When functioning as gRNA, the 5'-cap and 3'-CS sequence are occupied by the VP1-VP3 polymerase/capping enzyme complex. (B) Diagram of HBV preC-RNA and pgRNA transcripts. Both RNAs are ~3.5 kb and the preC-RNA has an additional ~30 nt at the 5'-end. The ε-elements required for packaging is labeled. The bottom cartoon schematic shows a proposed mechanism for the distinct functions of preC-RNA and pgRNA. The 5'-cap of preC-RNA is accessible for eIF4E to initiate translation. The 5'-cap of pgRNA is proposed to be sequestered by 5'-RNA elements or by the viral polymerase. (C) The flavivirus plus-strand RNA adopts two distinct conformations: the linear conformation promotes translation, and the circularized conformation suppresses translation and promotes RNA replication/packaging. The binding of viral polymerase NS5 to a 5'-cap proximal site in the circularized conformation is required for translation suppression and RNA replication/packaging. gRNA, genomic RNA; HBV, hepatitis B virus; pgRNA, pre-genomic RNA; preC-RNA, pre-core protein mRNA

selective encapsidation appear to be mechanistically coupled. We propose that the HBV pgRNA translation suppression and its selective packaging may be due to the inaccessibility of its 5'-cap and envisage a mechanism in which the distance between the 5'-cap the ε-element is critical for the potential cap sequestration in the HBV-Pol/pgRNA complex (Figure 4). One possibility is that the leader region of the pgRNA could facilitate RNA structure-based cap sequestration. A second possibility is that HBV-Pol may possess a cap-binding site similar to the above-mentioned rotavirus polymerase VP1 (protein-mediated cap-sequestration). It is also possible that

the binding of HBV-Pol may interfere with the subsequent assembly process of the translation machinery rather than the 5'-cap/eIF4E recognition.

Translation suppression by the binding of viral polymerase to the 5' proximal site of the plus-strand RNA has also been observed for flaviviruses. The genome of flaviviruses is an ~11 kb single-stranded positive-sense RNA encoding a single large polypeptide (C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5) that is co- and post-translationally cleaved into the mature viral proteins.^[19] In addition to the function as mRNA, the viral gRNA serves as the template for

RNA synthesis and is also selectively packaged into progeny viral particles.^[121] The gRNA of flaviviruses contains a 5'-cap and a conserved 3'-stem loop structure (3'-SL) instead of a polyadenylation signal.^[19,122] The highly structured 5'-UTR (~100 nt) and 3'-UTR (~400–700 nt) play important roles in flavivirus RNA translation and replication, such as the recognition by the viral RdRp NS5. It was shown that flavivirus RNA packaging requires the presence of a replication-competent NS5 polymerase.^[123] It was also found that the flavivirus gRNA adopts two distinct conformations to cope with its multi-functions: a linear conformation that promotes translation, and a circularized conformation that suppresses translation and promotes RNA replication/packaging (Figure 4C).^[124–126] gRNA circularization is mediated by long-range interactions between RNA elements in the terminal UTRs and the binding of NS5 is necessary for the translation suppression in the circularized gRNA (Figure 4C).^[127] The requirement of NS5 for flavivirus RNA packaging and NS5-mediated translation suppression is reminiscent of behaviors described above for other viruses and suggests the possibility of an RNA structure or NS5 dependent 5'-cap sequestration mechanism.

SUMMARY

The recent finding that RNA packaging by HIV-1 is strongly attenuated by cap exposure supports the hypothesis that cap dependent capture by the host RNA processing and translation machinery is a dominant negative determinant of packaging.^[25] Examination of the RNA packaging properties of other viruses that require 5' RNA capping for replication suggests a common overall mechanism for control of cap-dependent functions (trafficking/processing/translation) versus packaging fates. In each case examined, packaging appears to be promoted by processes that have the potential to hide or remove the 5'-cap. Thus, HIV-1 uses structural changes associated with heterogeneous transcriptional start site usage to modulate cap exposure; de-capped or noncapped RNAs are selectively packaged into retrovirus-like retrotransposons; and the binding of viral protein factors such as the polymerase in rotavirus, HBV, and flaviviruses, which is critical for viral RNA packaging, may be attributable to a direct cap sequestration function. We hypothesize that 5'-cap sequestration might be a general strategy used by viruses to inhibit capture by the cellular RNA translation machinery and thereby enable packaging of plus-strand RNAs as genomes or pre-genomes of progeny viruses.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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