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Idiosyncrasies of Viral Noncoding RNAs Provide Insights into Host Cell Biology

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Abstract
Like their host cells, many viruses express noncoding RNAs (ncRNAs). Despite the technical challenge of ascribing function to ncRNAs, diverse biological roles for virally expressed ncRNAs have been described, including regulation of viral replication, modulation of host gene expression, host immune evasion, cellular survival, and cellular transformation. Insights into conserved interactions between viral ncRNAs and host cell machinery frequently lead to novel findings concerning host cell biology. In this review, we discuss the functions and biogenesis of ncRNAs produced by animal viruses. Specifically, we describe noncanonical pathways of microRNA (miRNA) biogenesis and novel mechanisms used by viruses to manipulate miRNA and messenger RNA stability. We also highlight recent advances in understanding the function of viral long ncRNAs and circular RNAs.
INTRODUCTION
High-throughput RNA sequencing has broadened appreciation of the prevalence and diversity of noncoding RNAs (ncRNAs) expressed by pathogens and their host cells. Although these studies have advanced our observational understanding of infectious disease states, mechanistic details lag behind. Viruses are parasites that habitually exchange genetic material with their hosts. However, the limited size of viral genomes means that coding potential is carefully rationed. RNAs are less immunogenic than proteins and therefore provide an advantageous means to commandeer host cell functions. As a result, RNA and DNA viruses regularly express and optimize ncRNAs for their own benefit. Therefore, even though a viral ncRNA may exhibit characteristics typical of a particular host cell ncRNA, viral transcripts may have noncanonical functions. Investigating the complex interplay of viral ncRNAs and host cell activities has provided many insights into host cell biology.

The best-studied viral ncRNAs are produced by herpesviruses, which are a sizable family of vertebrate viruses characterized by large double-stranded DNA genomes and the ability to cause lifelong infections. Members of this virus family are notorious human pathogens, including herpes simplex virus 1 (HSV-1; oral and genital herpes), Epstein-Barr virus (EBV; mononucleosis and Burkitt's lymphoma), Kaposi sarcoma–associated herpesvirus (KSHV; Kaposi sarcoma), and varicella zoster virus (chicken pox and shingles). Herpesvirus-infected cells exist in two broadly defined states: the latent phase when the double-stranded circular viral genome is unobtrusively maintained in the host cell nucleus and the lytic phase when active replication produces new viruses. Because of their large genomes, complex life cycle, and medical relevance, the majority of well-studied viral ncRNAs, as well as the greatest number of ncRNAs per viral genome, have been described in herpesviruses.

Some of the most abundant viral ncRNAs are transcribed by RNA polymerase III (Pol III). Such RNAs include the cytoplasmic VA (virus-associated) RNAs from adenovirus and the nuclear EBER1 and EBER2 from EBV. VA RNAs inhibit protein kinase R to counteract the host cell antiviral defense (1, 2) and competitively block microRNA (miRNA) biogenesis by the RNase III enzyme Dicer (3, 4). Both VA RNAs and EBERs bind the autoantigen La, which recognizes the U-rich 3′ termini of RNA Pol III transcripts (5, 6). Beyond its binding partners La, RPL22, and AUF1, almost nothing is known about the function of EBER1 (7, 8). In contrast, the role of EBER2 is better understood; it recruits PAX5 to nascent transcripts emanating from the viral terminal repeat region to regulate EBV DNA replication (9), which is known to be critical for virally induced tumorigenesis. Detailed reviews of EBERs and VA RNAs are provided elsewhere (10–12).

In addition to discrete noncoding transcripts produced by viral genomes, cis-acting structures in RNA viral genomes and messenger RNAs (mRNAs) are required for viral replication and protein production. For instance, picornavirus cis-acting RNA structures present in the 5′ and 3′ nontranslated regions and open reading frame (ORF) together facilitate replication of the RNA viral genome by mediating interactions with viral trans-acting factors (13). Mechanisms for noncanonical translation of viral transcripts enable viruses to customize translation to their own needs. No virus is known to encode its own ribosome; yet, evolutionary pressure to optimize viral protein expression has driven the evolution of internal ribosome entry sites and frameshifting pseudoknots. These fascinating structures are discussed in depth in other reviews (13–15).

In this review, we highlight a selection of viral ncRNAs that have either an established function or the potential to provide insights into an important aspect of ncRNA biology (Table 1). As in previous reviews, we do not discuss the many viral ncRNAs of low abundance, which have been identified by high-throughput sequencing efforts but are as yet uncharacterized. We look forward to new mechanisms of host cell manipulation that may be uncovered by future studies.
| Table 1 | Viral ncRNAs, excluding miRNAs |
|---------|--------------------------------|
| Virus   | RNA | Viral family (subgroup) | Length (nt) | Associated protein(s) | Function | Reference(s) |
| Adenoviridae | VAII | Human adenovirus | 7,160 | III La, PKR, Dicer | Inhibition of PKR | 1,3,4,142 |
| Alphaherpesviridae | HSV-1 LAT | | ND | II ND | Repression of HSV-1 gene expression | 112,143 |
| Betaherpesviridae | MCMV 7.2-kb sisRNA | | ND | II ND | Promotion of progression from acute to persistent infection | 110 |
| Gammaherpesviridae | EBV EBER1 | | 167 | III La, L22, hnRNPD | ND | 5,7,9,109 |
| | EBV EBER2 | | 297 | II Variegale | ND | 109 |
| | EBV siRNA-1 | | 1,431 | II Sm, Ago2, HuR, hnRNPD | miRNA degradation | 62,145–147 |
| | EBV siRNA-2 | | 2,971 | II Sm, Ago2 n4, LmNA, hTRNP | ND | 62,145–147 |
| | HSV HSUR1 | | 2×10⁴ | II Sm, Ago2, HuR, hnRNPD | miRNA-mRNA adaptor | 62,145–147 |
| | HSV HSUR2 | | 2×10⁴ | II Sm, Ago2, HuR, hnRNPD | ND | 62,145–147 |
| | HSV HSUR5 | | 2×10⁴ | II Sm, Ago2, HuR, hnRNPD | ND | 62,145–147 |
| | HSUR3,-4,-6,-7 | | 10⁴–10⁵ | II Sm | ND | 145,148 |
| | KSHV PAN | | 5×10⁴ | II Sm, Ago2, HuR, hnRNPD | ND | 62,145–147 |
| | MHV68 tRNA | | 72–84 | III ND | ND | 152 |
| | Flaviridae | Flaviviruses | 280–700 | II Viral | ND | 48,145–147 |
| | Retroviridae | HIV-1 ASP RNA | | ND | ND | 88,89 |

Abbreviations: EBV, Epstein-Barr virus; HCMV, human cytomegalovirus; HIV-1, human immunodeficiency virus 1; HSV-1, herpes simplex virus 1; HVS, herpesvirus saimiri; KSHV, Kaposi sarcoma-associated herpesvirus; MCMV, murine cytomegalovirus; MDV, Marek's disease virus; MHV, murine gamma-herpesvirus 68; ND, not determined; PKR, protein kinase R.
VIRAL miRNAs ARE PROCESSED BY NONCANONICAL PATHWAYS OF BIOGENESIS

miRNAs are small, ∼22-nt ncRNAs that post-transcriptionally downregulate gene expression (16). A single miRNA can regulate hundreds of different mRNAs; indeed, they play roles in almost every cellular pathway. miRNAs are distinguished from other small ncRNAs by their biogenesis, evolutionary conservation, and function (17). Namely, miRNAs are processed from hairpin stem-loop structures by Drosha and Dicer RNase III–family enzymes, and they guide Argonaute (AGO) proteins—as part of the RNA-induced silencing complex (RISC)—to imperfectly complementary sequences in mRNA 3′ untranslated regions (UTRs) (18). The first viral miRNAs were identified in EBV (19). Since then, hundreds of viral miRNAs have been described (20)—with roles in viral replication, pathogenesis, and immune evasion (reviewed in 21, 22). Here, we highlight new insights into noncanonical pathways for viral miRNA production and novel methodologies for viral miRNA identification.

Canonical miRNA biogenesis begins with RNA polymerase II (Pol II) transcription of primary miRNAs (pri-miRNAs). These capped and polyadenylated transcripts can be several kilobases long and feature distinct secondary structure characteristics that allow recognition and cleavage by the Microprocessor complex, composed of the endonuclease Drosha and the RNA binding protein DGCR8 (23–25) (Figure 1). The excised ∼65-nt stem-loop, known as the precursor miRNA (pre-miRNA), is exported to the cytoplasm where it is further processed by Dicer to form one or two mature miRNAs (26–28). Viral miRNA biogenesis frequently deviates from this canonical miRNA production pathway as viruses often evolve creative methods to hijack host cellular machinery (reviewed in 29). Recently, even more examples of viral miRNA precursors transcribed by Pol III, or processed by Drosha- or Dicer-independent means, have been discovered (30–42). These unconventional mechanisms expand our conception of the miRNA biogenesis machinery and virus-host coordination.

The ideal human pri-miRNA substrate for Drosha comprises an ∼65-nt-long pre-miRNA hairpin featuring a 10-nt terminal loop, internal bulges in the stem (i.e., ∼11 nt or one helical turn apart), and flanking unstructured RNA sequences (24, 25, 43). Roughly half of all human pri-miRNAs also exhibit specific sequence motifs at the 5′ base and terminal loop of the hairpin that are required for efficient processing (44, 45). Bovine foamy virus (BFV) generates three mature viral miRNAs from a Pol III–transcribed bicistronic pri-miRNA cluster that harbors two hairpins separated by a single unpaired nucleotide (35, 36) (Figure 1c). How this pri-miRNA is processed is unknown; the dumbbell-shaped secondary structure should be a poor substrate for the Microprocessor because of the lack of unstructured regions at the base of each pre-miRNA stem. Mutational studies of the BFV pri-miRNA reveal that flanking sequences and local secondary structure contribute to proper expression of these BFV miRNAs, thereby suggesting secondary structural constraints similar to those for canonical pri-miRNA processing (35).

The first miRNA discovered in a single-stranded RNA virus came from a flavivirus, West Nile virus (WNV) (38). Before this discovery, one dilemma concerning miRNAs expressed by RNA viruses was how miRNAs could be excised without destruction of the RNA viral genome. To overcome this, flaviviruses produce a noncoding subgenomic flavivirus RNA (sfRNA) during viral replication, which is discussed later in this review. Bioinformatic interrogation and Northern blot analyses of viral ncRNAs in WNV-infected mosquito cells identified a miRNA-like small RNA, KUN-miR-1, processed from a pre-miRNA hairpin present in the 3′ UTR of the WNV genome (38) (Figure 1e). Some putative viral miRNAs are referred to as miRNA-like small RNAs because they have no known functions or evolutionary conservation and rarely fully follow the canonical miRNA biogenesis pathway. In WNV, knockdown experiments determined that
miRNA biogenesis pathways. In the canonical pathway, a capped and polyadenylated pri-miRNA undergoes cleavage by the Microprocessor complex. The ~65-nt pre-miRNA is transported from the nucleus to the cytoplasm, where it is subsequently cleaved by Dicer to produce an ~21-nt double-stranded product that features 2-nt 3' overhangs at both ends. The mature miRNA strand is then loaded into AGO, where it works as part of the RISC. Noncanonical viral miRNA biogenesis pathways include the following:

(a) HSURs are ncRNAs that are transcribed colinearly with a viral pre-miRNA hairpin. The Integrator complex processes the precursor by cleaving at the 3' ends of the HSUR and the pre-miRNA. (b) MHV68 encodes eight small ncRNAs known as TMERs. RNase Z cleavage at the 3' end of the tRNA-like structure releases the cotranscribed pre-miRNA hairpin. (c) BFV pri-miRNA harbors two pre-miRNA hairpins that bypass Drosha processing in an unknown manner. (d) HIV-1 has a miRNA-containing TAR RNA element at the 5' end of all HIV-1 transcripts. The miRNA is excised when a viral transactivator protein, TAT, interacts with stalled transcripts to allow Dicer processing of the stem-loop. (e) The 3' SL of WNV is recognized and processed by Dicer. (f) The 3' UTR of DENV-2 consists of several stem-loop structures, one of which associates with and is processed by AGO2 to release a miRNA.

Abbreviations: 3' SL, 3' terminal stem-loop; AGO, Argonaute; BFV, bovine foamy virus; DENV-2, Dengue virus 2; HIV-1, human immunodeficiency virus 1; HSUR, herpesvirus saimiri U-rich RNA; MHV68, murine gamma-herpesvirus 68; miRNA, microRNA; ncRNA, noncoding RNA; pre-miRNA, precursor miRNA; pri-miRNA, primary miRNA; RISC, RNA-induced silencing complex; TAR, trans-activation response; TMERs, tRNA-miRNA–encoded RNAs; UTR, untranslated region; WNV, West Nile virus. Figure adapted from Reference 128 and reused with permission under a CC-BY license.

Dicer-1 is required for KUN-miR-1 expression, providing evidence that it is a true miRNA (38). Dengue virus 2 (DENV-2)-infected mosquito cells similarly express a miRNA-like small RNA, DENV-vsRNA-5, which maps to a stem-loop located in the 3' UTR of the DENV-2 viral genome (37) (Figure 1f). DENV-vsRNA-5 expression is reduced by half in Dicer-1 or AGO1-depleted
DENV-2-infected mosquito cells but is even more reduced in AGO2-depleted cells. In addition, the mature and precursor miRNAs as well as a larger (240-nt) miRNA-containing transcript were present in RNA associated with AGO2 but not AGO1, which associates only with mature miRNA (37). These results strongly suggest AGO2 processing of this putative viral miRNA. In H5N1 influenza-infected human cell lines, RNA interference (RNAi) of Dicer, Drosha, or AGO2 significantly reduced expression of the miRNA-like small RNA miR-HA-3p only after AGO2 knockdown, but not after Dicer or Drosha, again implicating AGO2-mediated miRNA maturation (39). Whether these AGO2-dependent putative viral miRNAs follow a biogenesis pathway similar to the well-studied example of host AGO2-dependent miRNA processing of vertebrate miR-451 requires further study (46–48).

Subgenomic nonprocessive transcription is another method viruses exploit for miRNA generation. Human immunodeficiency virus 1 (HIV-1) possesses a 57-nt trans-activation response (TAR) RNA element, which folds into a hairpin at the 5’ end of all HIV-1 transcripts (42,49–51) (Figure 1d). The viral transactivator protein TAT interacts with TAR on nascent viral RNAs to promote HIV-1 transcription and replication (52, 53). Total small RNA sequencing and AGO2 high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation identified a miRNA-like viral small RNA, miR-TAR-3p, that maps to the HIV-1 TAR sequence. AGO-bound miR-TAR-3p is present at higher levels in infected 293T cells expressing a mutant virus engineered to block TAT-TAR transcriptional activation than those expressing wild-type HIV-1 (42). This phenotype was attributed to an increase in production of nonprocessive TAR transcripts from the mutant virus. Finally, investigation of Dicer-depleted and TAT-mutant cells revealed that TAT binding rearranges the TAR hairpin into a better substrate for Dicer processing. Overall, miR-TAR-3p is the product of short, paused, or prematurely stopped RNA Pol II transcripts that are bound by TAT to allow processing by Dicer (42).

Another strategy used by viruses to generate miRNAs involves inclusion of other viral ncRNAs in the pri-miRNA transcript. Six miRNA sequences appear immediately downstream of the herpesvirus saimiri (HVS) RNA Pol II viral ncRNA transcripts known as HSURs (herpesvirus saimiri U-rich RNAs) (41) (Figure 1a). HSURs resemble the Sm class of cellular small nuclear RNAs (snRNAs) that are essential for pre-mRNA splicing and histone pre-mRNA 3′ end processing (48,49). Cleavage at the 3′ ends of HSUR2, HSUR4, or HSUR5 sequences by the host Integrator complex releases a colinear downstream pre-miR-HSUR hairpin, each including two viral miRNAs (41). Alignment of pre-miR-HSUR sequences revealed a second processing signal called the miR 3′ box, which is also recognized and cleaved by the host Integrator complex (54). Therefore, the Integrator complex directly interacts with HVS pri-miRNA to excise pre-miRNAs by cleaving both at the 5′ and 3′ sides of the hairpin.

Murine gamma-herpesvirus 68 (MHV68) miRNAs are cotranscribed by Pol III as pre-miRNA stem-loops located immediately downstream of a transfer RNA (tRNA)–like structure. These transcripts are known as TMERs (tRNA-miRNA–encoded RNAs) (Figure 1b). The tRNA processing enzyme, RNase Z, cleaves at the 3′ end of the tRNA-like structure, releasing viral premiRNAs in a Drosha-independent manner (31, 32). Genetic studies using TMER knockout viruses provide evidence that these transcripts are multifunctional—the tRNA-like ncRNA plays an important role in infection and virulence comparable to that of the viral miRNA in vivo (55, 56). It will be interesting to see how those roles relate to the emerging research on tRNA fragments, which have been implicated in regulation of gene expression, apoptosis, and epigenetic inheritance (reviewed in 57, 58).

Viral miRNAs are detected primarily by high-throughput small RNA sequencing coupled with computational tools that predict pre-miRNA hairpins (19, 37–39, 41, 59). Identifying viral miRNAs in this manner has been challenging given that the vast majority (>98%) of sequencing...
reads map to the human genome and viral miRNAs can be moderately or lowly expressed compared to other abundant viral transcripts. The hunt for more viral miRNAs, particularly from retroviruses, has led to innovative bioinformatic and synthetic gene sequencing strategies (33, 34, 60). Candidate pre-miRNAs, identified by algorithms that predict RNA Pol III viral transcripts that form RNA hairpin secondary structures, were transiently expressed in cells and subjected to Northern blot analysis to confirm production of mature miRNA sequences. Using this approach, viral miRNAs produced by bovine leukemia virus and simian foamy virus were identified and characterized (33, 34). Discovery of five miRNAs expressed by four different papillomaviruses (PV) was accomplished using a new approach known as miRNA discovery by forced genomic expression or miDGE (60). In this methodology, PV genomes were fragmented, cloned into expression libraries, and transfected into cells. High-throughput small RNA sequencing reads obtained from these cells were mapped to the initial pool of PV genomes, and miRNAs were identified by the accumulation of sequencing reads at predicted pre-miRNA hairpins (61).

With the discovery of each new viral miRNA comes insight into the innovative approaches viruses use to manipulate cellular machinery: miRNAs are powerful gene regulators that are compact, an ideal situation for any virus. Conversely, it is in the best interest of a virus to defend itself against host miRNAs—an undertaking also accomplished by viral ncRNAs.

MODULATION OF HOST miRNA STABILITY AND TARGET SELECTION BY VIRAL ncRNAs

HSUR1 from HVS was the first example of an RNA that binds to host miRNA and, instead of undergoing RISC-mediated degradation, induces decay of the bound miRNA (62). Normally, specific binding to target mRNAs is conferred by the seed sequence of the miRNA (nucleotides 2–7) and results in RISC-mediated inhibition of translation, induction of mRNA decay by decapping and deadenylation, or both (16). HSUR1 binds to miR-27 family members in a unique fashion: In addition to base-pairing via the miRNA seed, HSUR1 exhibits extensive complementarity to the 3’ portion of the miRNA (62) (Figure 2a). This manner of interaction induces miR-27 degradation. Decreased levels of miR-27 in T cells cause prolonged T cell activation and correlate with virus-induced oncogenic transformation (63). The discovery of HSUR1-mediated miR-27 degradation was followed by two reports of other herpesviral RNAs that also selectively induce host miRNA degradation: the m169 RNA from murine cytomegalovirus (MCMV), which mediates degradation of host miR-27 family members, and a transcript from human CMV that targets host miR-17 family members (5). This target-directed miRNA degradation (TDMD) is believed to be a widespread cellular mechanism for regulating miRNA populations (64–66). Crystallization of AGO2-miR-27a-HSUR1 complexes has shed light on the molecular mechanism of TDMD. HSUR1 complementarity to the 3’ portion of miR-27a dislodges the miRNA 3’ end from its binding pocket in AGO2, thereby rendering the miRNA susceptible to host cell enzymatic attack (J. Sheu-Gruttadauria, P. Pawlica, J.A. Steitz, I.J. MacRae, et al., manuscript in preparation). Ongoing efforts to identify nucleotidyl transferases and exonucleases mediating TDMD have been challenging due to the redundancy and promiscuity of these cellular enzymes (65, 67). Bioinformatic discovery of novel TDMD effector RNAs awaits better definition of TDMD requirements beyond finding that such ncRNAs must be devoid of internal structure (65, 66, 68, 69).

The 116-nt HVS HSUR2 has evolved to mediate miRNA target selection (70). HSUR2 base-pairs with miR-142-3p and miR-16 (Figure 2b) as well as with ~70 host mRNAs. HSUR2 tethering of these two miRNAs destabilizes target mRNAs, which likely occurs via recruitment of RISC. mRNA targets of miR-142-3p and miR-16 modulate hematopoietic lineage development and the G1-S cell cycle transition, respectively. In the presence of HSUR2, additional downregulation of mRNA encoding proteins involved in cell cycle regulation (RB1), apoptosis (FAS), and
Figure 2
Modulation of miRNA stability and target selection by HSURs: schematized interactions between Sm-bound HSURs and host mRNAs. (a) HSUR1 binding to miR-27 dislodges the miRNA 3′ end from its pocket in the AGO protein followed by miRNA degradation by as-yet-unknown enzymes. (b) HSUR2 interaction with miR-142-3p and/or miR-16 recruits the AGO protein to target mRNAs—also tethered by HSUR2—for RISC-mediated mRNA decay. Abbreviations: AGO, Argonaute; HSUR, herpesvirus saimiri U-rich RNA; miRNA, microRNA; RISC, RNA-induced silencing complex.

the immune response (JAK1) is observed; these processes are relevant to viral maintenance during latency. It is unclear how HSUR2 targets multiple mRNAs; however, unpublished evidence suggests that different segments of HSUR2 base-pair with different mRNA targets (D. Cazalla, personal communication; see also 71). This example may represent a more general mechanism that cells use to degrade specific endogenous cellular mRNAs.

Deciphering the roles of HSUR1 and HSUR2 led to the discovery of two novel mechanisms of miRNA regulation, but the roles of the other five HSURs remain unknown. These advances highlight the potential for other viral ncRNAs to modulate miRNA activities and biogenesis in ways that have yet to be imagined.

FUNCTIONS OF VIRAL LONG ncRNAs REMAIN ELUSIVE
Long ncRNAs (lncRNAs) are arbitrarily defined as RNA transcripts longer than 200 nts that are transcribed by RNA Pol II and lack identifiable protein coding potential (72). According to their genomic positions relative to protein-coding genes, lncRNAs are categorized as antisense lncRNAs, bidirectional lncRNAs, enhancer RNAs, intronic lncRNAs, long intergenic ncRNAs, or transcribed pseudogene lncRNAs. In contrast to host lncRNAs that tend to be expressed in low copy numbers, viral lncRNAs can be among the most abundant transcripts in the cell. This disparity in expression levels hints at essential roles for lncRNAs during viral infection. High expression levels facilitate biochemical detection of these RNAs, yet deciphering their functions is far from easy.

In lytic KSHV-infected cells, the most abundant polyadenylated transcript is a nuclear lncRNA called PAN RNA. The best-studied aspect of PAN RNA is a triple-stranded 3′ end element, called
the element for nuclear expression (ENE), that robustly inhibits nuclear RNA degradation and is required to maintain PAN RNA at elevated levels (73–76). PAN RNA homologs in other gammaherpesviruses have been identified through bioinformatic searches for ENE structures (76). Loss of KSHV PAN RNA expression, either through knockout from the viral genome or antisense depletion of the transcript, results in misregulation of late lytic viral genes and host immune response genes (77, 78). Characterization of PAN RNA interaction partners has led to hypotheses that PAN RNA epigenetically regulates host and viral gene expression, promotes viral lytic reactivation, or sequesters host proteins (reviewed in 10, 79). Chemical probing of PAN RNA identifies three branched domains, but local structural details within each domain change depending on the biological source of PAN RNA: nucleus, cytoplasm, or virion (80). This structural variability suggests that PAN RNA may associate with, and potentially sequester (80) or concentrate (81), different protein partners during the viral lytic cycle. Delineating salient interaction partners and functions of PAN RNA will continue to illuminate how this viral lncRNA acts as a global regulator of the herpesvirus lytic phase.

During the latent phase, one of the few transcripts expressed from the HSV-1 genome is LAT (latency-associated transcript). Mutational and genetic ablation studies of LAT have implicated this viral lncRNA in preventing apoptosis (82), supporting neuronal tropism (83), establishing latency (84), promoting lytic reactivation (85), and regulating histone methylation (86, 87). More studies are needed to tease apart the temporal and spatial factors that allow this lncRNA to influence so many aspects of HSV-1 latency.

HIV-1, in addition to the full-length genomic RNA (gRNA) and spliced mRNAs, produces antisense RNAs (88, 89). One such transcript, the HIV-1 antisense protein (ASP) RNA, is a 2.6-kb, nuclear lncRNA transcribed from the 3′ long terminal repeat (LTR) U3 region of integrated proviral DNA (88). ASP RNA lacks a poly(A) tail and interacts with and recruits Polycomb Repressor Complex 2 (PRC2) to the HIV-1 5′ LTR. Subsequently, PRC2 trimethylates histone H3 lysine 27 (H3K27me3), which promotes nucleosome assembly and suppresses viral gene expression. Therefore, ASP RNA facilitates proviral latency through accumulation of the suppressive epigenetic mark H3K27me3 and reduction of proviral transcription (89). Although ASP RNA is readily detectable in chronically infected cell lines and latently infected cells from clinical samples (~5–25 copies per cell) (89), it has been hypothesized that the recruitment of PRC2 complexes to HIV-1 proviral loci might be mediated through other, as-yet-unknown lncRNAs (90).

The sometimes-contradictory list of phenotypes and functions associated with each viral RNA highlights how difficult it is to ascribe a mechanism of action to an lncRNA. Further complicating this is the fact that many lncRNAs may be multifunctional. Advances in biochemical and high-throughput techniques are needed to devise unbiased approaches to decipher the pertinent roles that viral lncRNAs play in infection.

**VIRAL TELOMERASE RNA IS ESSENTIAL FOR ONCOGENESIS**

Telomeres are repetitive sequences at the ends of chromosomes that prevent loss of genetic content through cycles of DNA replication. Telomeres are maintained by telomerase, consisting of two essential components: a telomerase reverse transcriptase (TERT) and an RNA molecule known as telomerase RNA (TR), which serves as a template for telomere repeat addition by TERT (91). Heightened telomerase activity has been detected in many cancers and contributes to transformation by maintaining cells in a proliferative state (92, 93).

Oncogenic viruses take advantage of telomeres and telomerase using different strategies. Examples include upregulation of host mRNA encoding TERT, which promotes survival of the infected cell (94), and integration of the viral genome into the host genome mediated by telomeric
Mutational analyses reveal regions important for vTR function. A comparison of chTR and vTR nucleotide sequences revealed additional nucleotides in the P2b region of the pseudoknot of chTR that reduce the ability of vTR to promote telomerase activity (100). Mutating the template region of TR (AU5 mutant) in the MDV genome prevents lymphomagenesis (102). Disruption of the p6.1 stem-loop also precludes tumor formation (103). Mutational analysis of p6.1 and template sequences were performed in the context of vTR. Abbreviations: chTR, chicken telomerase RNA; MDV, Marek’s disease virus; TR, telomerase RNA; vTR, viral telomerase RNA.

repeat sequences in the viral genome, which promotes prolonged infection of the host cell (95, 96). Marek’s disease virus (MDV), an alphaherpesvirus that causes lymphomas in chickens (97), produces its own viral TR (vTR), which complements the function of the chicken TR (chTR) (97–99). Interestingly, vTR, relative to chTR, elevates telomerase activity more efficiently within the cell (100). The increased efficiency of vTR is attributed to sequence variations in the P2 and P3 helices of the pseudoknot domain (Figure 3). Four nucleotides in the P2b stem of chTR, when inserted into vTR, diminish the capacity of vTR to promote telomerase activity (Figure 3).

The high transformation rate of MDV-infected cells, along with the precedent of heightened telomerase activity in cancer, has prompted investigation into the role of vTR in lymphomagenesis. MDV mutants that lack vTR do not efficiently induce lymphomas in their hosts but are able to replicate normally (101). Disrupting the interaction between vTR and chTERT by mutating the p6.1 stem of vTR (Figure 3) delays, but does not eliminate, the onset of lymphomagenesis (102). This suggests that elevated telomerase activity is important for the rate of transformation but is not essential for disease progression (103). Mutagenesis also revealed that an intact template...
region of vTR is necessary for transformation of T cells upon MDV infection (102) (Figure 3). The reduced rate of tumorigenesis resulting from a vTR template mutant virus infection revealed vTR as a potential therapeutic drug target: Vaccinating chickens with a vTR template mutant MDV produced an efficient, protective immune response (97, 102).

The possibility that vTR has roles independent of its interaction with TERT is supported by the finding that vTR interacts with the ribosomal protein RPL22 (103). RPL22 also interacts with human telomerase (104) and with the EBER1 ncRNA, which is produced by the oncogenic gammaherpesvirus EBV and implicated in tumorigenesis (7). Expression of EBER1, vTR, or chTR is required for relocalization of RPL22 from the cytoplasm and nucleoli to the nucleoplasm (7, 103). Furthermore, the interaction between EBER1 and RPL22 is important for proliferation (105). The relevance of the interaction between vTR and RPL22 in tumor progression upon MDV infection has yet to be addressed.

EBER2, which modulates lytic EBV replication by regulating latent gene expression (9), drives oncogenesis in MDV-infected cells lacking vTR (106). It is unclear whether the mechanisms by which EBER2 promotes oncogenesis are similar to those employed by vTR. Mutational analysis and the identification of additional vTR interacting partners will further the understanding of this process. The role of EBERs and vTR in cancer development and progression highlights the functional relevance of viral ncRNAs.

**VIRAL CIRCULAR RNAs AND STABLE INTRONIC SEQUENCE RNAs: PERVERSIVE, ABUNDANT, AND ENIGMATIC**

Alternative splicing of nascent pre-mRNAs differentially joins exons, resulting in RNA isoforms that may have different cellular functions or encode different protein variants. A form of alternative splicing called backsplicing joins a 3′ splice donor to an upstream 5′ splice acceptor to generate a covalently closed circular RNA (circRNA) (107). CircRNAs are remarkably stable due to the absence of exonuclease-susceptible termini. Most introns and intron fragments produced as a result of splicing are unstable; however, there are some notable exceptions including small nucleolar RNAs (snoRNAs) and stable intronic sequence RNAs (sisRNAs) (108, 109). snoRNAs function in the modification or processing of ribosomal RNAs, snRNAs, mRNAs, and tRNAs (reviewed in 108). Definitive biological functions for sisRNAs and circRNAs have yet to be elucidated, but since both classes of these ncRNAs are produced by viruses, they are potentially potent regulators of cellular function.

At least four viruses produce sisRNAs: human cytomegalovirus, MCMV, HSV-1, and EBV (109–112). EBV-encoded sisRNAs were discovered during analysis of a nuclear RNA library constructed from cultured EBV-infected B lymphocytes at the cancer-associated latency III stage. ebv-sisRNA-1 and -2 are abundant nuclear transcripts expressed from the W-repeat region of the EBV internal repeat 1 and are produced as a result of EBNA-LP (Epstein-Barr nuclear antigen leader protein) pre-mRNA splicing (109). Transformation of B cells by EBV requires EBNA-LP expression (113), and genetic mutation of ebv-sisRNA-1 reduces EBNA-LP expression and inhibits the transformation of B cells by EBV (114). ebv-sisRNA-1 and -2 interact with as many as 81 human proteins, 7 of which have been validated biochemically: FUS, hnRNP1A, hnRNP2C, hnRNP, hnRNPD/AUF1, HuR, and LIN28 (115). The EBV ncRNA EBER1, which also binds hnRNPD/AUF1 (9), may cooperate with ebv-sisRNA-1 to perturb the homeostasis of hnRNPD/AUF1-dependent mRNA regulation in EBV-infected cells. Interaction of the splicing repressor hnRNPL with ebv-sis-RNA may alter the nucleo-cytoplasmic distribution of hnRNPL, thereby influencing alternative splicing of host transcripts (116). Alternatively, association of ebv-sisRNA with hnRNPL might alter EBNA-LP pre-mRNA splicing to regulate sisRNA production.
Further functional investigations are needed to establish the implications of sisRNA interactions in EBV infection and disease.

circRNAs in EBV- and KSHV-infected cells were recently discovered by high-throughput RNA sequencing of ribosomal RNA-depleted and linear RNA-depleted (exonuclease RNase R-treated) libraries. At least 30 unique viral circRNAs were reported, including EBV-encoded circRNAs from the BART/RPMS1, BHLF1, and LMP-2 loci and KSHV-encoded circRNAs from the vIRF4 and PAN/K7.3 loci (117, 118). Investigation of KSHV circRNAs is still in its infancy.

EBV circRNAs are expressed in both latent and lytic stages, and some display extraordinarily high expression levels. Although mechanistic analyses of EBV circRNAs are as of yet lacking, the literature hints at potential functions. circBHLF1 may be the functional form of BHLF1 RNA that associates with the viral origin of replication, oriLyt, to facilitate lytic viral DNA replication (119). The EBV BART locus produces four abundant circRNAs: two nuclear intron-containing circBARTs and two exon-only circBARTs, which are found in both the nucleus and the cytoplasm. Host RNA helicases UAP56 and URH49, which contribute to host circRNA nuclear export (120), may likewise control export of viral circRNAs. The circBARTs and BART miRNAs are generated from pre-mRNA transcripts produced by the same locus. Like EBV BART miRNAs, circBARTs are present in all forms of EBV tumor latency; however, BART deletion studies showed that neither circBARTs nor BART miRNAs are essential for the maintenance of the EBV genome in cell culture. Nonetheless, circBART expression was detected in all EBV-associated tumor samples tested, implicating these ncRNAs in tumorigenesis.

The field of sisRNA and circRNA biology is nascent. circRNAs can sponge miRNAs, modulate RNA splicing, provide templates for cap-independent translation, and act in competition with their equivalent linear mRNA (reviewed in 121). Given the broad spectrum of latent and lytic genes from which these viral ncRNAs originate, their diverse patterns of expression, and their various subcellular distributions, viral sisRNAs and circRNAs may contribute to a wide array of functions. Additionally, the extreme stability of latency-associated and cancer-associated viral circRNAs enables their use as biopsy markers and diagnostic reagents (117, 118).

INHIBITION OF 5’ TO 3’ EXONUCLEASES BY STRUCTURED RNA ELEMENTS GENERATES VIRAL ncRNAs

An unusual mode of ncRNA production is employed by RNA viruses of the genus Flavivirus, which includes such arthropod-borne human pathogens as DENV, WNV, Zika virus (ZIKV), and yellow fever virus. An ∼300–700-nt-long sfRNA is generated by incomplete degradation of the gRNA by the cellular 5’ to 3’ exonuclease Xrn1 (reviewed in 122, 123). sfRNA is the most abundant viral RNA in flavivirus-infected cells.

The positive-sense gRNAs of flaviviruses possess a single ORF preceded and followed by highly structured UTRs (reviewed in 124). Some of the 3’ UTR structures, referred to as Xrn1-resistant RNAs (xRNAs), stall Xrn1 progression to generate stable sfRNAs. Most flavivirus gRNAs possess two adjacent xRNA structures (Figure 4), although virus gRNAs with only one xRNA have been identified (125). The 5’-proximal xRNA usually constitutes the major block to Xrn1 progression and thus demarcates the 5’ end of the most abundant form of sfRNA. Secondary structure mapping and mutational analysis of DENV sfRNA revealed a three-way junction in xRNA that is critical for halting Xrn1 (126). Subsequently, the crystal structures of xRNAs from Murray Valley encephalitis and ZIKV revealed an elegant RNA structure-based mechanism for Xrn1 stalling: The RNA fold organized around the three-way junction adopts a ring-like conformation with the 5’ end of the Xrn1-resistant structure passing through the ring (127) (Figure 4). The ring-like conformation is reinforced by a conserved pseudoknot and
Figure 4

Formation of noncoding sfRNA in flaviviruses. (a) gRNA from Zika virus, shown schematically (top), is degraded by Xrn1, which stalls at xrRNA structures. Stalling at xrRNA1 gives rise to the longest form of sfRNA (bottom). (b) A schematic depicts the three-dimensional structure of xrRNA1, which is boxed (dash lines) in sfRNA, as shown in a. The yellow box marks a unique ring-like structure through which the 5' end (blue) passes; this topological feature is believed to stall Xrn1 progression. The figure is not to scale.

Abbreviations: gRNA, genomic RNA; pk, pseudoknot; sfRNA, subgenomic flavivirus RNA; UTR, untranslated region; xrRNA, Xrn1-resistant RNA. Figure adapted from Reference 128.
two long-range interactions of 5′-proximal nucleotides with residues at the three-way junction. Although the primary and to some extent secondary structures of xrRNAs from distant branches of flaviviruses differ, based on nucleotide conservation and multiple compensatory changes within the long-range interactions, the tertiary fold is believed to be similar (128). Interestingly, subgenomic ncRNAs produced by incomplete degradation of the 3′ UTR of gRNA by cellular 5′ to 3′ exonucleases are found also in other animal and plant viral families (129–131). A crystal structure of xrRNA from a plant dianthovirus revealed a fold that is different from that of flaviviral xrRNAs (132). Nevertheless, both kinds of xrRNAs possess a common topological feature—a pseudoknot that creates a protective ring around the 5′ end of xrRNA to obstruct further degradation.

sfRNA is required for flavivirus cytopathicity and pathogenicity. It has been demonstrated to (a) dampen the antiviral activity of type I interferon (reviewed in 123), (b) interfere with cellular 5′ to 3′ RNA decay by inhibiting Xrn1 (133), and (c) inhibit the RNAi pathway in vertebrate and arthropod species, most likely by serving as a decoy substrate for Dicer (134, 135). Inhibition of the host interferon response appears to be, at least in some flaviviruses such as DENV-2, achieved by binding and inactivating cellular regulators of interferon-stimulated mRNA translation (136). Moreover, DENV-2 sfRNA binds TRIM25 to prevent TRIM25-mediated ubiquitination of the cytosolic RNA sensor RIG-I, thus averting viral RNA detection and interferon production (137). The RNAi inhibitory activity of sfRNA appears to be especially important for counteracting a potent antiviral RNAi response during the insect phase of the flavivirus life cycle.

CONCLUSIONS AND PROSPECTS

Powerful sequencing technologies present unprecedented opportunities to investigate questions in virology and RNA biology. The combined expertise of bioinformaticians and virologists will identify new viruses and viral ncRNAs, facilitate prediction of RNA structures, and enable increasingly penetrating transcriptomic analyses. Each newly discovered viral ncRNA is likely to have its own customized function. The peculiar ncRNA functions highlighted in this review are a small sampling of potential viral control mechanisms. Giant viruses, such as mimiviruses, are conceivably an untapped resource for viral ncRNA biology. These viruses typically have genomes larger than 200 kb and therefore possess substantial potential for expressing ncRNAs (138). Although giant viruses are studied primarily as infectious agents of single-celled eukaryotes, they have been isolated from human samples indicating potential roles in human health (139, 140). Finally, our understanding of the influence of RNA modifications on ncRNA function is only nascent (141). Post-transcriptional RNA modifications may prove to be potent modulators of the battle between viral ncRNAs and host cell defenses.

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**Errata**

An online log of corrections to *Annual Review of Virology* articles may be found at http://www.annualreviews.org/errata/virology