Balance and Stealth: The Role of Noncoding RNAs in the Regulation of Virus Gene Expression

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Abstract
In the past two decades, our knowledge of gene regulation has been greatly expanded by the discovery of microRNAs (miRNAs). miRNAs are small (19–24 nt) noncoding RNAs (ncRNAs) found in metazoans, plants, and some viruses. They have been shown to regulate many cellular processes, including differentiation, maintenance of homeostasis, apoptosis, and the immune response. At present, there are over 300 known viral miRNAs encoded by diverse virus families. One well-characterized function of some viral miRNAs is the regulation of viral transcripts. Host miRNAs can also regulate viral gene expression. We propose that viruses take advantage of both host and viral ncRNA regulation to balance replication and infectious state (for example, latent versus lytic infection). As miRNA regulation can be reversed upon certain cellular stresses, we hypothesize that ncRNAs can serve viruses as barometers for cellular stress.
INTRODUCTION

The “grandmother of punk,” American singer-songwriter Patti Smith, once said: “In life may you proceed with balance and stealth.” Although the context differs, this sentiment accurately describes the challenges that face viruses that undergo persistent infections. That is, they must have life cycles with balanced gene expression, maintained through stealthful mechanisms. Persistent viruses must optimize when, where, and how many virions are produced, while keeping host cells alive and avoiding the immune response. A major tool viruses use to accomplish this is the noncoding RNAs. Here we review the current understanding of both host and viral non-protein-coding regulatory RNAs (ncRNAs) in the regulation of virus gene expression.

Over the past decade there has been an exponential increase in our understanding of the role of ncRNAs in diverse areas of biology. There is an existing or emerging understanding of the mechanisms of action of several classes of ncRNA, including microRNAs (miRNAs) and long noncoding RNAs (lncRNAs). miRNAs are small RNAs (typically ∼22 nt) that repress gene expression by directing the RNA-induced silencing complex (RISC) to target mRNA transcripts, thereby inhibiting translation and/or decreasing the abundance of bound transcripts (1, 2). Unlike miRNAs, lncRNAs comprise a heterogeneous group of RNAs (typically >200 nt) whose biogenesis and mode of action are poorly defined. Regardless, some lncRNAs have been implicated in transcriptional and posttranscriptional control of gene expression (3). New classes of ncRNAs and associated functionalities will continue to emerge with the increased use of high-throughput RNA sequencing.

Like other fields, virology has been greatly impacted by ncRNAs. Both virus- and host-encoded ncRNAs contribute to the regulation of virus gene expression and the host response to infection. At least nine different virus families, spanning much of the Baltimore classification scheme, encode ncRNAs (4–8). ncRNAs are most prevalent in DNA viruses, but some retroviruses as well as negative- and positive-strand RNA viruses also give rise to ncRNAs (8, 9; R.P. Kincaid, Y. Chen, J.E. Cox, A. Rethwilm & C.S. Sullivan, unpublished observations). Although these ncRNAs can be diverse in terms of sequence, biogenesis, size, and structure, some shared activities are common. These include evasion of host defenses and regulation of virus gene expression (10). The role of ncRNAs in host defenses against virus infection has been extensively reviewed (6, 11–15). Here, we focus on ncRNAs that regulate virus gene expression.

MicroRNAs

miRNAs have been implicated in numerous virus-relevant processes, including the immune response, cell viability, and tumorigenesis (16–18). In the canonical pathway, miRNAs are derived from a primary transcript containing an approximately 70-nt stem-loop structure that is processed by the nuclear endonuclease Drosha and then the cytoplasmic endonuclease Dicer to give rise to the final ∼22-nt effector molecule (19). In general, miRNAs function as rheostats that serve to dampen gene expression (20). A single miRNA can have hundreds of different targets; conversely, a single transcript can have numerous docking sites for the same or multiple different miRNAs. In this manner, a complex web of potential interactions comes together to posttranscriptionally fine-tune gene expression (6).

miRNAs typically bind to the 3′ untranslated region (3′ UTR) of their target transcripts with imperfect complementarity. Commonly, this interaction is mediated in large part by the seed sequence (nucleotides 2–8), which directly binds to the target transcript via Watson–Crick base-pairing (21, 22). This results in translational repression, often accompanied by enhanced turnover of targeted transcripts. Less frequently, miRNAs can bind with perfect complementarity
PERSISTENCE AND LATENCY

Following de novo infection, some viruses can establish long-term persistent infections. Often this mode of infection is cell type–specific. Latency represents a specific subtype of persistent infection, defined as an alternative gene expression program that does not result in the production of virus (38). A defining characteristic of latency is that it can be reversed to allow full virus gene expression and production of virions (38). Persistent infections are maintained through modulation of both viral and cellular gene expression. Currently, there is a limited understanding of the mechanisms that control the maintenance of persistence and the switch to lytic infection. As many virus-associated diseases (for example, some cancers) are a consequence of persistent infection or escape from persistence, it is imperative to better understand the mechanisms of viral persistence. However, many common laboratory models of virus infection have been selected for the ability of the virus to undergo robust lytic infections and thus may be inappropriate for modeling persistence. Thus, future work on existing models is warranted, as is the development of new laboratory models of persistent infection.

(~22 out of 22 nt) and direct an irreversible cleavage of the target transcript, similar to siRNAs. This mode of regulation readily occurs in plants but is rarely seen in animals. However, some animal viral miRNAs direct cleavage of their antisense transcripts through this mechanism (23–27). It is interesting to note that miRNA silencing activity can be attenuated or even inverted in some circumstances, such as stress (28–33) (discussed below).

In most cases, any target of a miRNA is only subtly regulated by a single miRNA-target interaction. This suggests that the sum of numerous subtle regulatory events accounts for the profound biological effects often associated with miRNAs (34). As such, miRNAs can serve to balance “transcriptional noise” and maintain homeostasis (34–37). Thus, global perturbations of miRNA activity can be expected to reduce the tolerance of any given cell to various stressors.

To date, over 300 viral miRNAs have been discovered from diverse viruses (6, 38). Most of these viruses produce long-term, often lifelong, persistent infections (see sidebar, Persistence and Latency). miRNAs offer several advantages to a virus, including a small genomic footprint (most miRNAs can be encoded in less than 100 nt of genomic space) and presumed invisibility to the protein-based adaptive immune response. Although some viral miRNAs serve to block host defenses (including apoptosis and the adaptive and innate immune responses), an emerging concept is that many viral miRNAs regulate virus gene expression (10, 39, 40). This is of particular importance to those viruses that undergo persistent infections, as they need to optimize gene expression to allow for appropriate induction of the lytic, productive phase of infection. Below, we present specific examples whereby diverse viruses utilize viral or host miRNAs to control virus gene expression. For a comprehensive overview of ncRNA regulation of viral transcripts, refer to Table 1.

VIRAL MicroRNAs

Herpesviruses

Herpesviruses have large, double-stranded DNA genomes, typically greater than 100 kb, that code for numerous (>65) gene products. Herpesviruses can undergo latent infections in long-lived cells such as neurons or immune effector cells (the cell type depends on the infecting virus). Lytic infection proceeds via a temporally coordinated gene expression cascade from immediate early through late genes. Lytic infection can occur upon de novo infection or via exit from latency.
Table 1  Summary of viral and host miRNAs known to regulate viral transcripts

<table>
<thead>
<tr>
<th>Family</th>
<th>Virus(es)</th>
<th>miRNA</th>
<th>Target</th>
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<td>Herpesviridae</td>
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<td>miR-BART2</td>
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<td>miR-K12-10</td>
<td>V-IL-6, V-FLIP, V-cyclin, V-IRF3, LANA</td>
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<td>Human cytomegalovirus (HCMV)</td>
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<td>miR-UL112</td>
<td>IF1(^a), UL112/113, UL114, UL120/121</td>
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<td>Herpes simplex virus 1 and 2 (HSV-1 and -2)</td>
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<td>miR-H3, -H4</td>
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<td>miR-M7</td>
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<td>Early transcripts(^a)</td>
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<td>Simian agent 12 (SA12)</td>
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<td>Simian virus 40 (SV40)</td>
<td>miR-S1</td>
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### Table 1 (Continued)

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<td>miR-199a</td>
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<td>let-7</td>
<td>V-FLIP, V-cyclin, LANA</td>
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<td>M1</td>
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<td>miR-1650</td>
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<td>3′ mRNA</td>
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<td>miR-29a</td>
<td>Nef</td>
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<td>Simian immunodeficiency virus (SIV)</td>
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<td>Nef/U3 and R regions</td>
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<td>miR-142</td>
<td>3′ untranslated region</td>
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<sup>a</sup>Target transcripts that could reinforce persistent infection.
Pre-miRNA:
precursor miRNA that is cleaved by Dicer to form mature miRNAs

AntimiRs: synthetic oligonucleotides that inhibit a specific microRNA

Optimizing the switch from latent to lytic infection is likely crucial to the fitness of the virus and is controlled by various factors, including chromatin modifications, transcription, and the host adaptive immune response—all of which can be altered by various stressors (41–43). Recently, miRNAs have emerged as contributors to latent infection and the regulation of the switch to lytic infection in diverse herpesviruses (10).

Herpes simplex virus 1 (HSV-1) is the prototypic alphaherpesvirus. HSV-1 is associated with various diseases including cold sores, genital lesions, and lethal encephalitis. HSV-1 encodes more than six pre-miRNAs within the latency-associated transcript (LAT) (44, 45). Interestingly, the miRNAs and LAT are the only readily detectable transcripts during latency. Several HSV-1 miRNAs lie antisense (therefore with perfect complementarity) to the immunomodulatory ICP0 and ICP34.5 transcripts. As would be predicted, mutant viruses lacking HSV-1 miRNAs show increased expression of both ICP0 and ICP34.5 (44). ICP0 has also been implicated as necessary for reactivation (46–49). Importantly, infection of cultured Neuro2 cells at a low multiplicity of infection (MOI) with miRNA-mutant viruses resulted in increased viral replication. This phenotype was dependent on both MOI and cell type; infection of Neuro2 cells at a higher MOI or infection of NIH3T3 cells yielded no difference between wild-type and miRNA-mutant viruses (50). This suggests a possible role for the HSV-1 miRNAs in enforcing latency in vivo, although such a hypothesis awaits confirmation. Furthermore, the conditional nature of the increased replication phenotype offers a cautionary note for interpreting those negative studies in which miRNA mutant viruses show no apparent phenotype (25, 27).

Human cytomegalovirus (HCMV) is a prevalent human betaherpesvirus that is typically asymptomatic in healthy individuals. Infection of the immunocompromised individual or the unborn fetus can result in life-threatening illnesses and birth defects. Unlike other herpesviruses, the betaherpesviruses do not express miRNAs from one or a few clusters but rather have numerous distinct miRNA loci (at least 14) dispersed throughout the genome (51). Although multiple HCMV miRNAs can directly regulate various HCMV transcripts, the functional relevance of most of these interactions remains unknown (52, 53). The best-studied HCMV miRNA, miR-UL112, clearly regulates multiple immediate early genes (39, 54). Consequently, ectopic expression of miR-UL112 can negatively regulate virus replication (54). Grey et al. (39) were among the first to demonstrate that miR-UL112 can negatively regulate multiple viral transcripts. Furthermore, engineered mutant viruses (null for either miR-UL112 or one of its direct viral target sites, e.g., IE-1) have altered target viral protein levels (40). These studies were performed in fibroblasts where HCMV undergoes a persistent infection, continuously producing virions. It remains unknown what role HCMV miRNAs might play during latent infection; however, miR-UL112 is detectable in at least one model of latent infection (55). Furthermore, infection of a mouse model with a murine cytomegalovirus lacking two miRNAs resulted in less virus production in the salivary glands (56). The phenotype was dependent on both the initial viral titer and the genetic background of the mouse. It will be interesting to test a similar role for HCMV miRNAs in the establishment or maintenance of latency.

Kaposi sarcoma–associated herpesvirus (KSHV) is a gammaherpesvirus associated with rare B cell and endothelial tumors in immunosuppressed patients. KSHV encodes 12 pre-miRNAs that have a wealth of established host and viral target transcripts (6, 10, 57–61). Although latency is the typical default pathway for gammaherpesviruses, expression of the immediate early gene product RTA is sufficient to initiate the lytic replication cycle (62). At least three KSHV miRNAs can negatively regulate RTA expression via direct binding to its 3' UTR (59, 63, 64). Furthermore, several host targets of KSHV miRNAs have been identified that can affect RTA transcription (65–68). Whereas inhibition of individual KSHV miRNAs via antimiRs can lead to a decreased or increased propensity to initiate lytic replication, deletion of 10 of the 12 pre-miRNAs gives rise to viruses with
an increased propensity to undergo lytic activation (64, 67–69). Thus, a model emerges whereby multiple miRNA-target interactions (of viral and host origin) combine to reinforce latent infection. In addition to RTA, cross-linking immunoprecipitation (CLIP) studies have revealed several other viral targets of KSHV miRNAs, including both lytic (V-IL-6, V-FLIP, V-cyclin, V-IRF3) and latent (LANA) transcripts (57, 59). The benefit to the virus of this regulation remains unknown. However, there are at least two examples of seed conservation between a KSHV miRNA and two closely related rhesus gammaherpesviruses, termed RFHVM and RRV, which suggests that some of this regulation is likely evolutionarily conserved (70, 71). Additional viral transcripts that may be regulated by miRNAs were uncovered by screening a library of KSHV 3′ UTR reporters (72). A similar analysis that we conducted revealed an unexpected degree of regulation, with over 50% of the KSHV 3′ UTRs conveying significant negative regulation (73). This implies a possible global strategy of gene regulation.

The mechanisms accounting for this stage-specific regulation are mostly unknown, we have characterized one such example. This atypical regulation occurs through Drosha cleavage of pre-miRNAs that are positioned in the 3′ UTRs of Kaposin B and K12, as well as the coding portion of K12 (74). Drosha activity is high in latency, resulting in only a small portion of K12 and Kaposin B protein-coding transcripts escaping cleavage. However, during lytic infection, Drosha levels are reduced, with a consequent increase in the transcription of the K12 and Kaposin B loci. Because a positionally conserved pre-miRNA is found in the 3′ UTR of the RFHVM K12 transcript, we predict that this mode of cis regulation is likely evolutionarily conserved (71). We note, however, that much of the regulatory potential of the KSHV 3′ UTRs is independent of cis or trans effects of the KSHV miRNAs (73). Though well-studied for the RNA viruses, the possible widespread role of UTRs in DNA virus biology remains an exciting and understudied topic.

Epstein–Barr virus (EBV) is a gammaherpesvirus prevalent in the human population and associated with tumors, especially in immunosuppressed patients. EBV encodes at least 25 pre-miRNAs derived from one of two separate poly-pre-miRNA clusters (58, 75, 76). In the original description of EBV miRNAs, due to its antisense location, the transcript encoding the viral DNA polymerase (BALF5) was identified as a likely target of EBV miR-BART2 (76). This was later functionally validated by Barth et al. (26). Recently, CLIP strategies on EBV miRNAs have identified numerous host and a few viral target transcripts including LMP1, BHRF1, and EBNA2 (57, 77). Interestingly, no lytic transcripts (including BALF5) were identified as EBV miRNA targets, but it remains unclear whether this is due to the low levels of lytic transcripts that “leak” during latency or whether EBV miRNAs do not target lytic transcripts in a meaningful way (10). However, transfection of an antimiR against miR-BART6 leads to an approximately 2-fold increase in ZTA and RTA transcripts levels (78). As increased ZTA and RTA could promote lytic activation, these results are consistent with EBV utilizing miRNAs to optimize the latent/lytic switch. The authors propose that induction of ZTA and RTA is an indirect result of the global miRNA reduction resulting from downregulation of Dicer by miR-BART6 (although it remains possible that other targets could account for this phenotype) (78). Despite these data, a mutant EBV defective for miRNA production did not display increased markers of lytic infection compared with a recombinant virus engineered to express the full repertoire of EBV miRNAs (79). Thus, it remains unknown whether there exists a context in which EBV utilizes viral miRNAs to preferentially reinforce latent over lytic infection.

**Polyomaviruses**

Polyomaviruses (PyVs) are unrelated to herpesviruses in genome and structure, but they may share similar challenges in maintaining long-term persistent infections. These small DNA viruses are...
Autoregulation: regulation of a viral transcript through a viral gene product

prevalent in vertebrates, and at least 12 infect humans (80, 81). Although typically asymptomatic, in immunosuppressed patients some PyVs have a clear association with rare but serious diseases. These include Merkel cell carcinoma, progressive multifocal leukoencephalopathy, nephropathy, organ rejection in transplant patients, and trichodysplasia spinulosa (82–85). The prototypic PyVs include murine polyomavirus (MPyV) and simian virus 40 (SV40) (86). Both of these emerged as laboratory models due in part to their selected ability to undergo robust lytic infection in cultured cells. To date, virus-encoded miRNAs have been identified from at least six different PyVs (23–25, 27, 87). All are found antisense with perfect complementarity to the early transcripts that give rise to the multifunctional tumor antigens (T antigens). As would be predicted from their location, these miRNAs can direct siRNA-like cleavage of the early transcripts (23–25, 27).

Although there are undoubtedly some important host targets, these transcripts differ among the PyVs, because the seed regions of most PyV miRNAs are not shared. This does not rule out a possible convergence to regulate similar pathways, as has been suggested for KSHV and EBV (57). As in the herpesviruses, the genomic positions of the PyV miRNAs are often conserved. Consequently, the ability to negatively regulate the early transcripts is also conserved. Two lines of evidence support the importance of miRNA-mediated autoregulation of early gene expression. First, closely related SV40 isolates can have miRNAs with different seed sequences (and therefore different host targets) while preserving similar regulatory activity on the early transcripts (88). Second, the PyV-related bandicoot papillomatosis carcinomatosis viruses (BPCVs) also encode miRNAs that autoregulate the early T antigen transcripts. However, unlike PyV miRNAs, BPCV miRNAs are not located antisense to and do not bind with perfect complementarity to the early transcripts (89). These findings support evolutionary pressure to regulate T antigen transcripts via miRNAs, as opposed to an alternative model of “tolerating” some degree of negative regulation as a necessary consequence of the antisense genomic location.

Although the biological relevance of PyV miRNA regulation remains obscure, three studies implicate a role for the miRNAs in negatively regulating the viral copy number (27, 90, 91). Thus, two non–mutually exclusive models emerge whereby miRNA-mediated autoregulation of the T antigen transcripts contributes to either (a) lytic infection and/or (b) persistent infection (88). It has been observed that a substantial fraction of the early transcripts are subjected to miRNA-mediated cleavage in lytic models, which suggests a role for autoregulation (25, 88). However, we note that in some cell culture models, no difference in virus replication for miRNA-mutant viruses is observed (25, 88). There is also evidence that suggests a role for autoregulation in persistence of both BK virus and SV40. The BK virus miRNA can limit viral replication in a cultured cell model, and the SV40 miRNA affects virus copy number during long periods of in vivo infection (25, 90, 91). It is currently unknown whether PyVs maintain long-term persistence via true latency (that is, via alternative and reversible gene expression profiles). However, as for the herpesviruses, these data argue that PyV miRNAs play a role in persistent infection. As more is understood about PyV persistent infection, it will be interesting to determine whether the PyV miRNAs participate in any hypothetical herpesvirus-like switch to full-on lytic infection.

Retroviruses

Like herpesviruses and polyomaviruses, some retroviruses cause long-term infections and encode miRNAs. Retroviruses generally possess RNA genomes, but they have unique replication cycles involving integration of a DNA form of the genome (92). In some instances, this integrated provirus can become transcriptionally silenced. When this occurs in a reactivatable state, it represents a true latent infection. At least three diverse retroviruses encode miRNAs [bovine leukemia virus (BLV), avian leucosis virus subgroup J (ALV-J), and some foamy viruses (FVs) (8, 9; R.P. Kincaid,
Y. Chen, J.E. Cox, A. Rethwilm & C.S. Sullivan, unpublished observations), but some do not (8, 9, 93). The best characterized retroviral miRNAs include the BLV miRNA B4, which mimics the oncogenic host miRNA miR-29, and the African green monkey simian foamy virus (SFVagm) miRNAs S4 and S6, which mimic the host miRNAs miR-155 and miR-132, respectively. All three of these host miRNAs can be prosurvival and/or immunoevasive. Interestingly, the homologous genomic region coding for the majority of SFV miRNAs is sometimes lost when the prototypic virus is cultured ex vivo in cell culture (94–96). These results suggest a possible autoregulatory role for at least some retroviral miRNAs and raise the possibility that this regulation is advantageous in maintaining persistent infection in vivo (R.P. Kincaid, Y. Chen, J.E. Cox, A. Rethwilm & C.S. Sullivan, unpublished observations).

ATTENUATED MicroRNA REGULATION DURING STRESS

As multiple virus families utilize miRNAs to control viral gene expression, it is interesting to note that miRNA-mediated regulation can be attenuated or even reversed during certain forms of stress. Cellular stressors that can foster reduced miRNA activity include starvation, amino acid depletion, endoplasmic reticulum stress, oxidative stress, and detection of intracellular or extracellular pathogen-associated molecular patterns (PAMPs) (29–33). The transition of some herpesviral and retroviral latent infections into productive virus replication can be promoted by some of these same stressors (66, 97–103). Thus, it is possible that one mechanism by which stress induces virus production is by alleviating miRNA-mediated repression of viral and/or host transcripts. In such a scenario, any miRNA target that is capable of initiating a prolytic feed-forward loop (for example, RTA in KSHV) could contribute to context-specific virus induction. Although it awaits validation, such a model implies that viruses may use miRNA-mediated regulation as a barometer for gauging cellular stress (Figure 1).

HOST MicroRNAs

From a viral perspective, virus-encoded miRNAs seem to offer several advantages, including the ability to stealthily regulate numerous host and viral transcripts while occupying relatively little genomic space. Therefore, it is striking that many viruses, especially those with positive- and negative-strand RNA genomes, do not encode miRNAs. Even some DNA viruses with nuclear life cycles, including some of the papillomaviruses (PVs), do not encode miRNAs (104, 105). It is likely that the life cycles of these viruses do not require the fine-tuning regulation provided by miRNAs, or that they obtain equivalent regulation via alternative strategies. One such strategy used by diverse viruses is to take advantage of host miRNAs. As discussed below, host miRNA-mediated regulation of virus gene expression can lead to consequential biological effects.

The PVs are small viruses with circular, double-stranded DNA; they are the causative agents of benign warts and several human cancers (106). Two reports (107, 108) claim that several alphapapillomavirus types (6, 16, 18, 38, 45, 68) encode miRNAs. However, these reports have not been independently verified and suffer from a lower level of experimental verification than is currently the norm for the field. Thus, it is not widely accepted that PVs encode miRNAs. What is clear is that at least one PV, human papillomavirus 31 (HPV-31), does not encode its own miRNAs (104, 105).

PVs can cause long-term persistent infections, whereby the genome is maintained as an episome in undifferentiated keratinocytes (109). Only upon cellular differentiation is the full repertoire of viral proteins expressed (109). Gunasekaran & Laimins (105) found in a raft culture model of keratinocyte differentiation that 93 host miRNAs were differentially expressed in the presence of the HPV-31 genome. One of these miRNAs, miR-145, directly targeted the
polycistronic early viral transcripts that can give rise to multiple different viral proteins (105). This regulation has functional relevance, as forced expression of miR-145 results in reduced viral genome amplification upon cell differentiation (105). In this way, HPV seems to manipulate host miR-145 levels to restrict genome amplification in undifferentiated cells, while allowing for full virus gene expression in differentiated cells. Thus, HPVs may use host miRNAs to the same ends as the PyVs and other viruses use autoregulatory viral miRNAs.

In addition to viruses altering host miRNAs to regulate virus gene expression, some viruses have altered tissue distribution due to preexisting differences in host miRNAs. The North American eastern equine encephalitis virus (EEEV) is an alphavirus that can be transmitted to humans via mosquito vectors (110). Although the case mortality rate is high, humans are generally considered to be a dead-end host; birds are the major vertebrate hosts of this arbovirus (111). There are direct target sites for the host miRNA miR-142-3p in the 3′ UTR of EEEV (112). Through the use of overexpression and inhibition strategies, it was demonstrated that miR-142 restricts EEEV replication. Interestingly, miR-142 expression is abundant only in hematopoietic cells, suggesting that this mechanism accounts for the lack of replication of EEEV in certain cell types such as macrophages and dendritic cells (112). In a mouse model, a mutant virus that is resistant to miR-142 regulation restores replication in macrophages (112). This gives rise to increased
interferon-α/β serum levels, and the virus is consequently less virulent (112). Even though mosquitoes do not express miR-142, the genomic region surrounding the docking sites for miR-142 is important for replication in the mosquito. The authors interpret this as evidence that this region of the UTR has redundant functions in the arbovector, and they suggest that this phenomenon may contribute to the positive selective pressure required to maintain these miRNA target sites (112). An alternative, non–mutually exclusive model is that miR-142-mediated regulation in the avian hosts could be advantageous to the virus, perhaps by allowing evasion of the innate immune response while somehow still permitting high enough levels of viremia. Thus, viruses may use miRNAs not only to coordinate with the differentiation and stress status of infected cells but also to limit replication in particular tissues.

In the past 10 years, there has been an ongoing debate involving miRNA regulation of HIV. In the midst of the controversy are contradicting reports that HIV encodes several miRNAs (60, 93, 113–116). Early reports based on low-throughput small-RNA sequencing and computational prediction technologies have not been recapitulated by other labs (60, 113). Thus, until it is independently verified that HIV encodes bioactive miRNAs with relevant functionality, caution is warranted toward any claims that HIV makes miRNAs. Additionally, HIV has been suggested to use host miRNAs to regulate its transcripts (117, 118). However, Whisnant et al. (113) recently demonstrated by using CLIP technologies that the HIV genome is largely resistant to host miRNA regulation. This study verified that a small subset of the published miRNAs do in fact directly contact a small fraction of HIV transcripts, but questions were raised as to whether this contact is sufficient for meaningful bioactivity (113). It is worth noting that a lncRNA, NEAT1, has also been shown to regulate HIV gene expression (119). Further studies are warranted to determine the effects of host ncRNAs on the HIV life cycle.

A SUGGESTED ROLE FOR HOST MicroRNAs AS A DIRECT ANTIVIRAL DEFENSE

In principle, host miRNA–mediated negative regulation of viruses falls into one of three categories: (a) a natural host defense against specific viral pathogens, (b) a gene regulation tool employed by the virus, or (c) a consequence of the laboratory system used that does not occur during natural infection (120). Several papers have published models whereby miRNAs act as a direct defense against specific pathogens (121–123). However, we note that others have questioned this interpretation because in some instances these host miRNAs are not expressed in a physiologically relevant context (120, 124, 125). This criticism is supported by experimental evidence demonstrating that some miRNAs are not at a sufficient copy number for bioactivity, or are not expressed in the appropriate host or cell type during natural infection (120, 124, 125). Any model whereby host miRNAs serve to directly combat a specific virus will have to account for the inability of these viruses to evolve resistance. This is especially problematic given that only a single nucleotide change can be sufficient to abolish miRNA-mediated suppression (126). As discussed above, miRNA-mediated negative regulation can be advantageous to the virus because diverse viruses encode their own autoregulatory miRNAs. Indeed, some miRNA-mutant viruses replicate better in cell culture and/or in vivo models (25, 27, 50, 90, 91). Yet, these viral miRNAs are maintained in the wild, and their functionalities are typically conserved among divergent viruses (10, 53, 70, 75, 127). This underscores the reality that standard laboratory assays sometimes do not recapitulate the proviral advantages conveyed by miRNAs during natural infection.

As new data emerge, it may be worth revisiting some of the early reports claiming direct miRNA antiviral effectors. In a notable study, Lecellier et al. (128) proposed that host miRNAs can be a direct antiviral defense. They demonstrated that miR-32 can repress primate foamy virus 1 (PFV-1;
Aptamer: typically an oligonucleotide (can be a protein) that binds to a specific target molecule.

note that PFV-1 now refers to prototype foamy virus 1) accumulation through a docking site located within the coding region of ORF2 that is also the 3’ UTR of all the remaining PFV-1 mRNAs (128). Although this was argued as a miRNA-based antiviral defense, it remains to be determined whether this interaction occurs to a meaningful degree in vivo and, if so, whether it is disadvantageous to the fitness of the virus. Importantly, the recent discovery that PFV and other SFVs encode highly expressed, evolutionarily conserved miRNAs (R.P. Kincaid, Y. Chen, J.E. Cox, A. Rethwilm & C.S. Sullivan, unpublished observations) demonstrates that some miRNA regulation is clearly advantageous to overall PFV fitness.

OTHER CLASSES OF VIRAL NONCODING RNAs

Decades before the discovery of miRNAs, virus-encoded ncRNAs were expected to play an important role in the infectious cycle (14). Several different viruses, including herpesviruses and adenoviruses, encode robust amounts of longer ncRNAs (~160 to >1,000 nt). Unlike miRNAs, these ncRNAs likely represent a hodgepodge of different mechanisms of action, and as such, a full understanding of how they function is still lacking. Two of these ncRNAs for which recent insights have been reported include the adenovirus virus-associated (VA) RNAs and the KSHV polyadenylated nuclear RNA.

VA RNAs are encoded by the lung- and gastrointestinal tract–tropic adenoviruses. These are among the best studied and oldest known viral ncRNAs. These ∼165-nt RNAs are transcribed by RNA polymerase III during the lytic cycle to 10^7 – 10^8 copies/cell, ranking among the most numerically abundant RNAs (14, 129). Both primate and human adenoviruses can express up to two VA RNAs (130). VA I RNA functions like a natural aptamer that stoichiometrically binds to and inhibits protein kinase R (PKR) (131). Thus, this represents an important counterdefense used by the virus to evade the antiviral response. Additionally, VA I inhibition of PKR indirectly promotes viral gene expression by limiting translational repression associated with PKR activation. Interestingly, less than 1% of the VA RNAs is processed into miRNAs. Despite the inefficient processing, VA-derived miRNAs are the most abundant miRNAs (~10^6 copies/cell) detected in a cell undergoing lytic infection (129). However, these miRNAs are likely not important during lytic infection. Unlike VA-null viruses, which are attenuated for virus production by 1–2 orders of magnitude, engineered mutant viruses that retain the larger VA structure but produce miRNAs with altered seeds behave like wild-type viruses (129). Although much less studied, adenoviruses can also produce a persistent infection (132, 133). Recently, it was shown in a cell culture model of persistence that the VA-derived miRNAs are readily detectable and account for ∼2.7% of the total RISC-associated RNAs (132, 134). Thus, it remains formally possible that VA miRNAs are important during persistent infection or in other unknown contexts, but this possibility has not yet been demonstrated (Figure 2).

KSHV encodes a 1.1-kb ncRNA called the polyadenylated nuclear RNA (PAN). PAN is expressed during lytic infection and is the most abundant viral transcript produced, topping out at 10^6 – 10^7 copies/cell (135). Although polyadenylated, PAN is nuclear and has an atypical 3’ end structure that renders it resistant to nucleolytic turnover (136, 137). Until recently, no functions for PAN had been reported. However, in the past few years, several exciting reports have been published. Knockdown and knockout studies show that PAN is important for virus gene expression (138, 139). Importantly, PAN is required for expression of RTA, the master lytic switch protein (140). The published mechanism that can account for this is that PAN is a lncRNA that alters viral chromatin (139). Very recently, a small fraction of PAN has been shown to be associated with translationally active ribosomes (141). Though only a small fraction, this still would potentially account for some of the most abundant virally derived peptides made during infection (141). The
Figure 2
Model: role of virus-associated (VA) I RNA in adenovirus infection. (a) VA I RNAs are structured, highly abundant RNAs encoded by some primate viruses (including human adenoviruses). During lytic infection, VA I RNAs stoichiometrically bind to protein kinase R (PKR), preventing its activation. This limits the cellular antiviral response and prevents translation inhibition, thus allowing viral gene expression (128). (b) VA I RNA, and the related VA II RNA in some adenoviruses, can be inefficiently processed into miRNAs. These miRNAs are made during lytic infection but have also been detected in cell models of persistent infection (130). It is therefore possible that the VA RNA-derived miRNAs function to maintain persistent infection, but such a model remains untested. Abbreviation: RISC, RNA-induced silencing complex.

WHAT ARE THEY GOOD FOR?
With our current understanding of the impact of miRNAs on viral gene expression, miRNAs represent a plausible avenue of antiviral therapeutics. Notably, human clinical trials are already underway for therapeutics that target miR-122 to limit hepatitis C infection. Hepatitis C uses miR-122 to positively regulate virus copy number (142, 143). The success of this work suggests that targeting virally encoded miRNAs could also be a viable treatment strategy when the delivery barrier of miRNA therapeutics can be overcome. Others have engineered multiple host miRNA docking sites to restrict infection by gene therapy vectors (144–146). This approach shows promise in limiting oncolytic viruses to tumor tissues. A similar approach has been developed to restrict species specificity to laboratory model animals of highly pathogenic avian influenza (147). Finally, in cases where viral genome copy number is limiting but miRNA copy number is high, viral miRNAs may be useful as biomarkers of disease. For example, this method has been proposed for early detection of a herpesvirus associated with a fatal disease in elephants (148, 149).

SUMMARY POINTS
1. Diverse viruses, including DNA viruses and retroviruses, encode miRNAs.
2. Many viral miRNAs target host and/or viral transcripts as a mechanism to regulate virus gene expression.
3. miRNA regulation plays an important role in regulating persistent infection.
4. Host miRNAs are unlikely to be a direct defense against specific viral transcripts.
5. Viruses may use miRNAs as barometers to measure stress levels in host cells.
6. Longer ncRNAs are emerging as important players in virus infection.
7. Manipulating miRNA regulation shows promise in optimizing viral gene therapy vectors.

FUTURE ISSUES
1. New studies of animal models are needed to establish the role of ncRNAs in viral persistence in vivo.
2. Determining the full repertoire of virus families that encode miRNAs will help us understand known viral miRNA functions.
3. Uncovering alternative mechanisms of regulation for persistent viruses that do not encode miRNAs is clinically important.
4. Entire new classes of viral and host ncRNAs relevant to infection likely await discovery.
5. Viral ncRNAs offer new therapeutics and biomarkers strategies.

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