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Viral and cellular messenger RNA targets of viral microRNAs

Bryan R. Cullen
Department of Molecular Genetics & Microbiology, Center for Virology, Duke University Medical Center, Durham, NC 27710

Abstract

Given the propensity of viruses to co-opt cellular pathways and activities for their own benefit, it is perhaps not surprising that several viruses have now also been shown to use virally encoded microRNAs to reshape the cellular environment to their benefit. In particular, microRNAs are expressed by the various members of the herpesvirus family of viral pathogens during both the latent and productive phases of their replication cycles. Emerging data suggest that viral miRNAs may be particularly important for regulating the transition from latent to lytic replication and for attenuating potentially inhibitory host antiviral immune responses.

Viral miRNAs were first reported by Pfeffer et al. in 20041, who described five miRNAs expressed by the γ-herpesvirus Epstein-Barr Virus (EBV) in infected human B cells. Subsequent work has revealed miRNAs encoded by all herpesviruses examined so far. EBV is now known to encode at least 23 miRNAs2,3 while the distantly related human γ-herpesvirus Kaposi's Sarcoma-Associated Herpes Virus (KSHV) encodes 123–5. Similarly, the β-herpesvirus human cytomegalovirus (hCMV) encodes 11 miRNAs5,6 while the human α-herpesvirus Herpes Simplex Virus Type 1 (HSV-1) encodes at least six miRNAs 7,8. (These numbers describe the known pre-miRNA precursors encoded by each virus. A pre-miRNA may give rise to a single mature miRNA or may give rise to two, of which one is expressed predominantly.) Several miRNAs have also been identified in the simian γ-herpesviruses Rhesus Rhadino Virus (RRV)9 and Rhesus Lymphocryptovirus (rLCV)2, the murine γ-herpesvirus 68 (MHV68)5, in murine cytomegalovirus (mCMV)10,11 and in the avian α-herpesviruses Marek's Disease Virus (MDV) Types 1 and 212,13.

In contrast to herpesviruses, other virus species apparently encode either one or two miRNAs (primate polyomaviruses and human adenovirus) or no miRNAs at all[5,14–17]. Viruses that have an RNA genome, including retroviruses and flaviviruses, have been reported to lack any viral miRNAs5,[17], although this result remains somewhat controversial for HIV-1[18]. The paucity of viral miRNAs in the RNA viruses examined thus far may be partly explained by the fact that excision of a pre-miRNA stem-loop by the host miRNA processing enzyme Drosha[19] would result in viral genome degradation. Moreover, most RNA viruses—as well as DNA viruses belonging to the poxvirus family—replicate in the cytoplasm, away from the nuclear microprocessor complex. It is less clear why miRNAs appear to be rare in nuclear DNA viruses that are not members of the herpesvirus family. It seems possible that this relates to the characteristic ability of herpesviruses to establish long-term, latent infections. Avoidance of host immune responses is perhaps particularly critical during latent infection, and viral miRNAs are not only non-antigenic but may also provide an ideal tool to attenuate host immune responses by down-regulating key cellular gene products. Moreover, miRNAs may provide a mechanism to regulate viral entry into and/or exit from latency[20]. In contrast, other DNA virus families normally establish productive infections that often result in the rapid death of the infected cell due to viral pathogenesis or host cytotoxic immune responses. MiRNAs, which act at the mRNA level, may be of less benefit during lytic replication as the targeted protein product may have a half-life that

approaches, or even exceeds, the viral life cycle. On the other hand, viral miRNAs could be effective inhibitors of cellular mRNAs that are induced during infection, and it will be interesting to see if additional viral miRNAs, encoded by DNA viruses outside the herpesvirus family, emerge in the future.

Expression of viral miRNAs

Cellular miRNAs are generally transcribed by RNA polymerase II as part of one arm of a stem-loop structure contained within a longer capped, polyadenylated primary miRNA (pri-miRNA) precursor[19]. pri-miRNA precursors are cleaved by Drosha to excise ~60 nt pre-miRNA hairpin intermediates, which are exported to the cytoplasm and processed by Dicer to generate mature miRNAs that are then loaded into the RNA induced silencing complex (RISC). There is currently no evidence to suggest that any vertebrate virus encodes novel miRNA processing factors or RISC components, and it therefore appears that viral miRNAs are generally transcribed and processed just like cellular miRNAs and, moreover, that RISCs programmed by viral or cellular miRNAs are functionally equivalent (Fig. 1). A minor exception arises in the case of the miRNAs expressed by MHV68 and by human adenovirus (hAD), as these are initially transcribed by RNA polymerase III[5,16]. While the processing steps involved in MHV68 biogenesis remain to be fully elucidated, it appears likely that the mature MHV68 and hAD miRNAs are excised by Dicer and loaded into RISC normally.

A key characteristic of DNA viruses is that gene expression during productive replication is temporally regulated so that viral proteins can be sub-divided into immediate early, early and late species. Immediate early gene products are generally regulatory proteins; early proteins are more diverse but are often involved in genome replication or in regulating host immune responses; while late proteins are usually structural. γ-herpesviruses, in particular, also express viral proteins during latency that play roles in episome maintenance, cell growth regulation, and immune avoidance. The question then is whether expression of viral miRNAs is also temporally regulated. For most herpesviruses, this is presently unclear, as the viral miRNAs were cloned either exclusively from latently infected cells or fairly late in the productive replication cycle. However, evidence suggests that some viral miRNAs are active during latency while others are more important during productive replication. For example, in the case of KSHV, all 12 viral miRNAs are found in a cluster that is transcribed as a single primary miRNA precursor during latent infection. However, during the transition to productive replication, a viral lytic promoter is activated that lies 3' to 10 of the KSHV miRNAs but 5' to 2 miRNAs. As a result, while the expression of 10 of the KSHV miRNAs is largely unaffected by activation of productive replication, the expression of two of the KSHV miRNAs is substantially induced[5,21].

In the case of hCMV, the 11 known viral miRNAs were all identified in productively infected cells[5,6], and the majority of these viral miRNAs appear to be expressed with "early" kinetics, i.e., their expression is dependent on viral immediate early transcription factors, and it remains unclear which hCMV miRNAs are expressed during latency. On the other hand, in the case of HSV-1, four viral miRNAs appear to be expressed primarily during latency, one exclusively during productive replication and one miRNA during both[7,8]. Finally, in the case of the polyomavirus SV40, viral miRNAs are expressed with late kinetics[14]. A full appreciation of the functions of viral miRNAs will certainly require a more detailed understanding of how their expression is regulated during the viral life cycle.

Although many viral miRNAs have now been identified, we have only a limited understanding of their functions. There are currently no published reports examining the *in vivo* phenotypes of viral mutants specifically lacking individual viral miRNAs, and only a small number of mRNA targets have been described (Tables 1 and 2). In principle, one can

imagine that viral miRNAs might have evolved to down-regulate cellular mRNAs and/or viral mRNAs (Fig. 1). Cellular mRNA targets might include transcripts encoding proteins involved in host innate or adaptive immunity or, more generally, in cell cycle regulation or signaling. Viral mRNA targets might include transcripts involved in regulating the transition from latency to lytic replication (or vice versa) or immediate early viral gene products that need to be eliminated at later stages in the viral life cycle, due to toxicity or because they provide targets for host cytotoxic T-cells[14,20]. Although still limited, known viral miRNA targets already provide examples belonging to almost all of these potential categories.

Viral mRNA targets of viral microRNAs

The first paper to describe viral miRNAs also provided the first indication of a viral mRNA target for a viral miRNA. Specifically, one of the five EBV miRNAs described by Pfeffer et al.[1], called miR-BART2, was found to lie antisense to the EBV DNA polymerase gene *BALF5* and was proposed to inhibit DNA polymerase expression by inducing cleavage of the cognate mRNA. Although the evidence supporting partial inhibition of EBV DNA polymerase expression by miR-BART2 is clear[22], the functional significance of this inhibition is not currently known. However, it is possible that inhibition of EBV DNA polymerase expression may promote entry into latency by reducing viral genome amplification early after infection. Recently, Hussain et al.[23] reported that a miRNA encoded by the insect DNA virus *Heliothis viriscens* ascovirus (HvAV) also acts to downregulate expression of the viral DNA polymerase. Unlike miR-BART2, this HvAV miRNA does not lie antisense to the viral DNA polymerase gene and has only moderate homology to this proposed mRNA target, yet a reduction in DNA polymerase mRNA expression was again observed. The report of two unrelated viral miRNAs, each of which reduces the expression of mRNAs encoding the cognate viral DNA polymerase, suggests the possibility of convergent evolution.

A second example of a viral miRNA that is transcribed antisense to a viral mRNA, and induces degradation of that mRNA, is provided by the simian polyomavirus SV40. SV40 encodes a single pre-miRNA stem-loop that is expressed exclusively as a late gene product[14]. The viral miRNAs derived from this stem-loop lie antisense to the early viral mRNAs that encode the SV40 T antigens, viral transcription factors that, among other things, induce late viral gene expression. These SV40 miRNAs, which show perfect complementarity to the T antigen mRNAs, induce their cleavage and degradation and reduce T antigen expression late in the SV40 life cycle. Evidence has been presented showing that epitopes derived from SV40 T antigens are targets for cytotoxic T-lymphocytes, and the effect of these viral miRNAs is therefore to partially protect SV40-infected cells from T cell killing[14].

Another example of viral miRNAs regulating genes to which they are antisense has been reported in HSV-1 and the related HSV-2[8,24]. During latency, HSV-1 expresses a set of five miRNAs called miR-H2 through miR-H6. One of these miRNAs, miR-H2-3p, lies antisense to the viral immediate early gene *ICP0* and has been shown to downregulate ICP0 protein expression[8]. Surprisingly, miR-H2-3p does not, however, induce ICP0 mRNA degradation despite being fully complementary. While the molecular basis for this phenomenon is currently unclear, others have also reported examples of miRNAs or siRNAs that reduce the expression of mRNAs bearing perfectly complementary targets primarily by inhibiting their translation[25,26].

In addition to miR-H2-3p lying antisense to *ICP0*, HSV-1 miR-H3 and miR-H4 also lie antisense to the gene encoding the pathogenicity factor *ICP34.5* and, based on genetic data, were proposed to inhibit ICP34.5 expression in latently infected neurons[8]. This hypothesis

has now been validated for the related virus HSV-2, which encodes a miRNA closely similar to miR-H3 called miR-I[24]. miR-I reduces ICP34.5 protein and mRNA expression when overexpressed in HSV-2 infected cells in culture. A final example of an HSV-1 miRNA that targets a viral mRNA is provided by miR-H6, which was shown to downregulate expression of the HSV-1 ICP4 protein[8]. miR-H6 does not lie antisense to the *ICP4* gene but miR-H6 does show extensive complementarity to ICP4 mRNA, including the entire miRNA sequence extending from position 2 to 8 of miR-H6, referred to as the miRNA "seed" sequence. Full mRNA complementarity to the miRNA seed is generally, but not invariably, required for translational inhibition[19].

In total, it therefore seems that four of the six known HSV-1 miRNAs are acting to downregulate viral mRNAs in latently infected cells. The combined action of miR-H2-3p and miR-H6, which downregulate the HSV-1 immediate early proteins ICP0 and ICP4 respectively (Table 1), may increase the likelihood of HSV-1 entering latency and/or inhibit the transition from latent to lytic replication[8]. The inhibition of ICP34.5 expression by miR-H3 and, potentially, miR-H4 is more difficult to explain, as ICP34.5 is a pathogenicity factor that blocks activation of the host antiviral factor PKR and inhibits autophagy [27,28]. It is possible that inhibition of ICP34.5 expression shields latently infected neurons from the severe cytopathic effects induced by a full-blown HSV-1 productive replication cycle and, indeed, HSV-1 mutants lacking the *IPC34.5* gene are much less neurotoxic[28].

Another example of a viral miRNA downregulating a critical immediate early protein is provided by hCMV miR-UL112-1, which downregulates expression of the viral IE72/IE1 protein by targeting two partially complementary sites located in the 3' untranslated region (3' UTR) of IE72/IE1 mRNAs20,29. This observation prompted the proposal that herpesviruses in general might use miRNAs to regulate the expression of viral proteins that can trigger the transition from latency to productive replication6. While this hypothesis remains far from proven, the observation that latent HSV-1 miRNAs downregulate expression of the immediate early proteins ICP0 and ICP48, as noted above, and recent data showing downregulation of the KSHV Rta and Mta/ORF57 immediate early proteins by viral miRNAs expressed during KSHV latency (P. Konstantinova and B. R. Cullen, unpublished observations) is certainly consistent with this proposal.

A final example of a viral gene product being downregulated by viral miRNAs is provided by the EBV LMP1 protein, whose expression has been reported to be downregulated by the three EBV miRNAs miR-BART1-5p, miR-BART16 and miR-BART17-5p[30]. LMP1 is a cytoplasmic signaling molecule, expressed during EBV latency, that is capable of inducing cell growth and transformation. However, overexpression of LMP1 can result in growth inhibition and elevated levels of apoptosis[30]. It is therefore possible that the role of these miRNAs is to ensure an optimal level of LMP1 expression during EBV latency.

Cellular mRNA targets of viral miRNAs

In principle, viral mRNA targets for viral miRNAs should be easier to identify than cellular mRNA targets. If a viral miRNA lies antisense to a viral mRNA, then this suggests an obvious potential target, although not all viral genes lying antisense to a viral miRNA are in fact downregulated by that miRNA[31]. Even if the viral miRNA acts on a partially complementary mRNA, this should be easier to identify given the far smaller viral genome size when compared to the host cell genome. In principle, one might predict that viral miRNAs might have evolved to be fully complementary to a host cell mRNA encoding a particularly troublesome host defense factor; however, no fully complementary cellular mRNA targets for viral miRNAs have been identified so far. Instead, viral miRNAs appear

to inhibit the translation of cellular mRNAs bearing partially complementary sites, i.e., viral miRNAs apparently function just like cellular miRNAs[19] (Table 2).

An extreme example of this is seen with the KSHV miRNA miR-K12-11, which shares an identical seed region with the cellular miRNA miR-155 and appears to downregulate an identical, or nearly identical, set of target mRNAs[32,33]. The most fully characterized of these is the mRNA encoding BACH1, which contains several targets for both miR-K12-11 and miR-155 in its 3' UTR. BACH1 is a transcriptional suppressor, and the significance of this downregulation for KSHV replication remains unclear. In fact, even though several human genes downregulated by both miR-K12-11 and miR-155 have been identified[32,33], the reason why miR-K12-11 has evolved to phenocopy miR-155 remains unclear. Overexpression of cellular miR-155 is, however, associated with B-cell transformation and it is possible that miR-K12-11 contributes to B cell transformation by KSHV[33]. Interestingly, the avian α-herpesvirus MDV-1 encodes a miRNA that also functions as an ortholog of miR-155[34] while EBV, although it does not itself encode a miR-155 equivalent, induces endogenous miR-155 expression in infected B cells[35]. It therefore appears that downregulation of specific cellular genes by either miR-155 itself, or by viral orthologs of miR-155, may facilitate the replication of a range of different herpesviruses.

Another cellular gene downregulated by KSHV miRNAs is Thrombospondin-1 (THBS1), a cellular protein involved in facilitating cell-to-cell adhesion that has been reported to have anti-proliferative and anti-angiogenic activity[36]. THBS1 expression is downregulated in Kaposi's Sarcoma (KS) tumors, and angiogenesis is a hallmark of KS. Samols et al. 36 observed that THBS1 mRNA was downregulated in cells engineered to express KSHV miRNAs and also showed that THBS1 translation is inhibited by several KSHV miRNAs, in particular by miR-K12-6-3p, which shows miRNA seed complementarity to two sites in the THBS1 mRNA 3' UTR. It therefore seems possible that downregulation of THBS1 by KSHV miRNAs contributes to the development of KS *in vivo*.

An obvious prediction is that viral miRNAs might downregulate cellular mRNAs that encode factors involved in mediating host antiviral immune responses, and the next three cellular targets satisfy this expectation. One reported target for hCMV miR-UL112-1 is MICB, a natural killer (NK) cell ligand that plays a key role in NK cell killing of virus-infected cells[37]. The proposed target for miR-UL112-1 in the 3' UTR of the MICB mRNA is unusual in that it does not display complete seed homology to miR-UL112-1, and this may represent a case where extensive homology to the central and 3' part of a miRNA compensates for incomplete seed homology37. In any event, cells expressing miR-UL112-1 were found to express less cell surface MICB and to be resistant to NK cell killing *in vitro*. Conversely, cells infected with a mutant form of hCMV lacking miR-UL112-1 showed enhanced levels of cell surface MICB, and were killed more effectively by NK cells, when compared to cells infected with wild-type hCMV. Interestingly, MICB function is also inhibited by the hCMV UL16 protein, suggesting that hCMV UL16 and miR-UL112-1 may be acting synergistically to protect infected cells from the NK arm of the human immune system[37]. Recently, it was reported that cellular miRNAs, including miR-93, also target the 3' UTR of the MICB mRNA via sites that partly overlap with, but are distinct from, the site targeted by miR-UL112-1 [38]. While these cellular miRNAs are not similar in sequence to viral miR-UL112-1, it nevertheless seems that this viral miRNA is mimicking the function of a subset of cellular miRNAs and thereby exerting a similar protective effect against NK cell killing. As noted above, miR-UL112-1 has also been reported to downregulate the viral IE72/IE1 immediate early protein in hCMV-infected cells (Table 1), thus providing the first example of a viral miRNA that targets both viral and cellular mRNAs.

A second example of a viral miRNA downregulating an innate immune response is provided by EBV miR-BART5, which has been reported to downregulate a pro-apoptotic protein called “p53 up-regulated modulator of apoptosis” or PUMA[39]. Depletion of miR-BART5 in EBV-infected nasopharyngeal carcinoma cells was found to trigger elevated levels of apoptosis mediated by PUMA, thus suggesting that miR-BART5 may act to shield EBV-infected epithelial cells, as well as EBV-transformed cells, from apoptotic elimination.

The final known example of a cellular gene product downregulated by a viral miRNA is provided by CXCL-11, also called I-TAC, an interferon-inducible chemokine that acts as an attractant for T-cells. The mRNA encoding CXCL-11 is downregulated by EBV miR-BHRF1-3, which is expressed at high levels in many B cell tumors induced by EBV[40]. As CXCL-11 has been shown to have antitumor activity in animal studies, this report raises the possibility that downregulation of CXCL-11 by miR-BHRF1-3 may shield EBV-infected B cells from cytotoxic T cells *in vivo*.

Conservation of viral microRNAs

The specificity of a given miRNA can be changed by as little as one or two changes in sequence, especially in the miRNA seed region[19], and one might therefore predict that viral miRNAs might be subject to rapid evolutionary drift. Conversely, if a given viral miRNA significantly enhances viral replication, then one might expect that miRNA to be conserved across significant evolutionary distances. Finally, if a viral miRNA targets viral mRNAs, one might expect co-evolution to occur, while, if a viral miRNA targets a cellular mRNA, then one might expect the evolution of the viral miRNA sequence to be restricted.

In fact, analysis of miRNAs encoded by different members of the herpesvirus and polyomavirus families has so far revealed very little sequence conservation. One exception arises in the case of EBV and its simian relative rLCV—seven of the 16 miRNAs expressed by rLCV turn out to be very similar to EBV miRNAs[2]. As EBV and rLCV are thought to have diverged ~13 million years ago, this suggests a strong evolutionary pressure for retention of the same miRNA sequence, especially as sequences closely adjacent to the mature miRNAs, e.g., in the terminal loop of the pre-miRNA intermediate, were found to have diverged significantly[2].

In contrast, other related viruses, e.g., KSHV and RRV or MDV-1 and MDV-2, show no miRNA sequence conservation[9,12], although the genomic location of the miRNAs encoded by these viruses is conserved. Conservation of genomic location, but lack of sequence similarity, is also seen with the simian polyomavirus SV40 and its human relatives BK Virus and JC Virus, which all express miRNAs that target mRNAs encoding viral T antigens[14,15]. This finding could suggest that the sole function of the miRNAs present in these viruses is to target these viral mRNAs[15]. However, as viral miRNAs that do not have any known viral mRNA targets also tend to maintain the same genomic location, even when their primary sequences have diverged[2,9,12], it is also possible that it is simply easier to select favorable sequence changes in a pre-existing miRNA stem-loop than to generate a novel stem-loop *de novo*, i.e., it remains possible that these diverse polyomavirus miRNAs also target cellular mRNAs for downregulation. Moreover, because two viral miRNAs have divergent sequences does not necessarily imply different functions. Two distinct miRNAs, encoded by two different viruses, could, for example, target two different targets in the same mRNA 3’ UTR, or perhaps target two different gene products that act at different steps in the same host metabolic pathway, thus leading to a similar phenotype. Until we have a greater understanding of the mRNA targets for viral miRNAs, and some idea of their *in vivo* functions, the conservation, or lack of conservation, of viral miRNAs is therefore not readily interpretable.

Despite our still limited knowledge of viral miRNA function, the large number of miRNAs encoded by diverse members of the herpesvirus family, and their high-level expression during latent infections, does suggest that these small non-coding RNAs are playing a key role in regulating viral pathogenesis *in vivo*. In particular, it will be important to test the hypothesis that herpesvirus miRNAs expressed during latency help maintain the latent state[8,20], using viral mutants and/or antisense reagents. In any event, it now seems possible that antisense reagents specific to particular viral miRNAs could significantly attenuate herpesvirus-induced diseases in humans, if they can be delivered effectively to infected cells *in vivo*.

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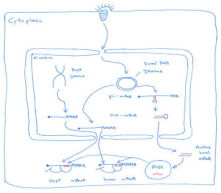

Fig. 1.

After infection by a DNA virus, the viral circular DNA genome is transcribed in the cell nucleus to give both pri-miRNAs and mRNAs. The pri-miRNA is processed by host factors in the nucleus to yield the pre-miRNA intermediate, which is then exported to the cytoplasm where the mature viral miRNA is generated. RISCs programmed by viral miRNAs can then inhibit expression of viral and/or cellular mRNA in the infected cell cytoplasm.

Table 1

Viral mRNA targets of viral miRNAs

Virus	Viral miRNA	Gene target	Function
EBV	miR-BART2	*BALF5*	DNA polymerase
EBV	miR-BART16, miR-BART17-5p, miR-BART1-5p	*LMP1*	Signaling molecule
SV40	miR-S1	*T antigens*	Early proteins
hCMV	miR-UL112-1	*IE72/IE1*	Immediate early protein
HSV-1	miR-H2-3p	*ICP0*	Immediate early protein
HSV-1	miR-H6	*ICP4*	Immediate early protein
HSV-2	miR-I	*ICP34.5*	Pathogenicity factor
HvAV	miR-1	*ORF1*	DNA polymerase

Known viral mRNA targets of viral miRNAs. See text for detailed discussion.

Table 2

Cellular mRNA targets of viral miRNAs

Virus	Viral miRNA	Gene target	Function
KSHV	miR-K12-6-3p(+ others)	*THBS1*	adhesion molecule/angiogenesis inhibitor
KSHV	miR-K12-11	*BACH1 (+ others)*	transcriptional suppressor
hCMV	miR-UL112-1	*MICB*	NK cell ligand
EBV	miR-BHRF1-3	*CXCL-11*	chemokine/T-cell attractant
EBV	miR-BART5	*PUMA*	pro-apoptotic