

New roles for large and small viral RNAs in evading host defences

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Abstract | It has been known for decades that some clinically important viruses encode abundant amounts of non-coding RNAs (ncRNAs) during infection. Until recently, the number of viral ncRNAs identified was few and their functions were mostly unknown. Although our understanding is still in its infancy, several recent reports have identified new functions for viral microRNAs and larger ncRNAs. These results so far show that different classes of viral ncRNAs act to autoregulate viral gene expression and evade host antiviral defences such as apoptosis and the immune response.

A fundamental change has occurred in our understanding of the mammalian transcriptome. Estimates suggest that up to 50% of the human and up to 70% of the murine genome is transcribed^{1,2}, altering previous notions that the majority of the mammalian genome is transcriptionally inert. Additionally, on the order of 70% of all transcripts overlap in the sense or anti-sense orientations³. Because many of these transcripts are expressed at low abundance, there is some debate as to the relevance of these observations. However, it is clear that this expanded view of the transcriptome opens the possibility for new modes of transcriptional and post-transcriptional control of gene expression⁴. New classes of abundant non-coding RNAs (ncRNAs) defined by common mechanisms of biogenesis and function continue to be identified. These include the microRNAs (miRNAs) that function to bind and modulate the ability of an mRNA to be translated (BOX 1). Taking shape is a picture of the mammalian transcriptome that has striking similarities to viruses with DNA genomes (DNA viruses).

Like their mammalian hosts, some DNA viruses transcribe a majority of their genomes, have overlapping transcripts and encode for abundant ncRNAs. Several families of DNA viruses encode ncRNAs that are among the most abundant transcripts detected during infection. These viruses have been associated with various diseases

ranging from minor respiratory illness to cancer and birth defects. Thus, deciphering the functions of these ncRNAs would not only help to better understand the viral infectious cycle but might be of potential clinical relevance.

Broadly speaking, viral ncRNAs can be divided into two classes: miRNAs, which have had such a reverberative effect that they are deserving of their own category, and everything else. For the purposes of this article, I will refer to the 'everything else' as the long ncRNAs (lncRNAs) because, so far, those

described range in size from approximately a hundred to a few thousand nucleotides in length — significantly longer than miRNAs.

Viral miRNAs

Recent studies on the functions of viral-encoded miRNAs suggest that at least some of these viral ncRNAs will function to evade host responses that are detrimental to infection (BOX 2). In 2004, Pfeffer and colleagues published a seminal paper that conclusively showed that a virus encodes a miRNA⁵. Additionally, host-encoded miRNAs have been shown to have a role in viral-relevant processes such as apoptosis, immune response and tumorigenesis. In hindsight, it seems logical that viruses would use miRNAs. From a viral perspective the advantages are obvious: miRNAs are non-immunogenic, take up a small amount of genomic space and are powerful regulators of gene expression. There are now over 120 viral miRNAs that are known⁶, mostly from the large DNA genome herpesvirus family, with an additional few being identified from the small DNA genome tumour viruses. As with host miRNAs, their functions are mostly unknown but recent headway has been made. New studies show that viral miRNAs evade the host innate immune response, regulate viral gene expression and possibly contribute to viral-mediated tumorigenesis.

Box 1 | MicroRNAs

MicroRNAs (miRNAs) are small regulatory RNAs that were discovered approximately 13 years ago through forward genetic studies in the nematode *Caenorhabditis elegans* (for reviews see REFS 31,32). For the most part, miRNAs function by binding to complementary sequences in the 3' UTRs of mRNAs, repressing translation and thereby blocking protein expression. Less commonly, miRNAs can bind to target mRNAs with perfect complementarity, and direct cleavage of the mRNA^{33,34}. Recently, Vasudevan and colleagues have shown that some miRNAs can actually induce an increase in protein expression, a process that is dependent upon cell-cycle arrest³⁵. Because a single miRNA can potentially regulate hundreds of mRNA targets, miRNAs can be thought of as nodal regulators that function to regulate entire networks of genes that are involved in a particular biological process. MiRNAs are generated as longer primary transcripts (pri-miRNAs) containing a ~90 nucleotide (nt) hairpin that undergoes a series of endonucleolytic processing steps into a ~70 nt hairpin precursor (pre-miRNA), and eventually into the final ~22 nt effector molecule that is bound by the miRNA-induced silencing complex (miRISC) (reviewed in REF. 36). To date, over 500 human miRNAs have been identified, most with unknown function. However, understanding of miRNA function is progressing rapidly. In general, a list of potential mRNA targets is generated using computational algorithms or exogenous expression and cDNA expression microarray analysis. If a subset of the targets identified regulate the same biological process (for example, apoptosis or cell-cycle regulation), then this approach can serve to provide direction towards understanding the function of an miRNA.

Box 2 | Host response to viral infection

Innate immune response

In mammals, the innate immune response occurs early after infection and is the primary immune response before the adaptive response can be mounted. The innate response can be an intracellular reaction of the infected cell or recognition by effector lymphocyte cells that are specialized to attack viral-infected cells. The effector cells of the innate response have germline-inherited receptors that have not undergone somatic recombination.

Adaptive immune response

The adaptive immune response is mediated by cells specialized to recognize antigens that are specific to a particular pathogen. The main effector cells target infected cells by means of receptors that have undergone somatic rearrangement and mutation. The adaptive response allows for great specificity and the ability to mount a faster and more robust defence against future infection.

Interferon

(IFN). A cytokine produced in response to pathogen infection and a key component of the innate intracellular immune response. Cells responding to IFN signalling express hundreds of genes, many of them specific to countering viral infection.

Protein kinase R

(PKR). An IFN-inducible kinase that can be activated by cues of viral infection, such as double-stranded RNA. Phosphorylation of PKR substrates creates a cellular milieu that is less conducive to viral infection, in part by preventing translation.

Apoptosis

Programmed cell death — an organized process in which the cell kills itself following detection of various stressors (including, sometimes, viral infection).

Natural killer cell

(NK Cell). An effector cell of the innate immune response; a circulating lymphocyte that is specialized to recognize positive and negative cell surface ligands. Infected cells trigger an aberrant pattern of receptors that activate the NK cell to directly kill the infected cell or to secrete cytokines to recruit other types of immune effector cells to the site of infection.

Major histocompatibility complex class I

(MHC class I). The MHC class I presents short peptides on the surface of cells that are recognized by effector cells of the adaptive immune system.

Cytotoxic T cells

(CTLs). Effector cells of the adaptive immune response that encode receptors to a specific peptide presented by MHC class I molecules. Binding to a specific MHC class I-bound peptide activates CTLs to kill the infected cell and to release cytokines to recruit other components of the immune response.

MicroRNA

(miRNA). A small ~22 nucleotide RNA that binds to and generally represses protein expression of specific mRNAs. MiRNAs have been implicated in the adaptive and innate immune response.

Viral long non-coding RNA

(Viral lncRNA). A term used in this paper to describe the longer RNAs (greater than 150 nucleotides) that are encoded by several families of DNA viruses. Some of these have been implicated in preventing different components of the innate immune response.

including viral infection. Cells infected with HCMV strains that are mutant for miR-UL112 have higher levels of MICB and are more susceptible to killing by co-cultured NK cells. Given the lack of an animal model for HCMV infection, it is unclear what role this function of miR-UL112 will have during infection *in vivo*. However, targeting MICB is probably crucial for successful HCMV infection *in vivo* because the viral protein UL16 reduces cell-surface expression of MICB by the incorrect localization of MICB to the endoplasmic reticulum or golgi⁹. Thus, HCMV uses both a protein gene product and a miRNA to target the same host protein. These findings suggest that one way to gain insight into some of the >100 viral miRNAs with unknown functions might be to focus on cellular pathways that are already known to be targeted by each respective virus. For example, this double strategy might be particularly important for some viral targets, such as those of the immune response, in which enhanced or redundant blockage of function is essential for productive infection.

KSHV miR-k12-11 and host miR-155 regulate a common set of mRNA targets. Kaposi's sarcoma-associated herpesvirus (KSHV) is the causative agent of Kaposi's sarcoma (KS) — a highly vascularized skin lesion — and several B-cell disorders. KSHV-associated disease is found predominantly in immunocompromised individuals such as those with advanced-stage AIDS. KSHV infection is associated with increased cellular hyperproliferation and cytokine secretion, and numerous viral proteins have been described with a role in these phenomena. Recently, the Renne and the Cullen groups have each independently shown that the KS miRNA miR-k12-11 and host miR-155 can regulate a shared set of target mRNAs^{10,11}. Both miRNAs share an identical seed sequence¹² (that is, nucleotides at the 5' end of the miRNA, typically positions 2–7), which has been shown to be a crucial determinant in binding specific mRNA targets. Using exogenous expression of the viral miRNA followed by microarray and computational sequence-matching analysis, each group generated a list of shared mRNA targets — some of which might have functions relevant to KSHV-associated disease, including apoptosis, cell-cycle regulation and innate immunity. Because aberrant expression of miR-155 is associated with lymphomagenesis and altered cytokine secretion, the findings of Gottwein *et al.* and Skalsky *et al.*, that KSMiRK12-11 is a functional orthologue of miR-155, suggest a possible role for a viral

HCMV encodes a miRNA that interferes with the host innate immune response. Human cytomegalovirus (HCMV) is a member of the herpesvirus family that generally forms a benign, lifelong infection in its hosts. However, HCMV can induce birth defects when passed to the unborn fetus and can cause life-threatening illness in immunocompromised individuals. Natural killer (NK) cells are components of the innate immune response that are activated following recognition of various cellular ligands, the expression of which is altered by viral infection (BOX 2). Activated NK cells can directly kill infected cells or secrete cytokines that activate other immune cells at the site of infection. Thus,

NK cells are a circulating first line of defence in controlling viral infection until an adaptive immune response can be mounted. HCMV and murine cytomegalovirus (MCMV) encode multiple proteins that use different strategies to prevent NK-cell activation; evading the activated NK-cell response is particularly important to successful cytomegalovirus infection⁷. Recently, Stern-Ginossar and colleagues have shown that HCMV uses yet another mechanism to evade NK-cell activation by a miRNA⁸. The miRNA miR-UL112 downregulates the expression of MICB (major histocompatibility complex class I polypeptide-related sequence B), a NK-cell ligand that is upregulated by cellular stresses,

miRNA in tumorigenesis. One unanswered question concerns the role of the remaining sixteen 3' nucleotides in determining target specificity; that is, will the 5' seed sequence be the major determinant of target specificity for endogenous expressed miRNAs, as seems to be the case in these exogenous expression studies? Interestingly, the use by KSHV of a pre-existing host miRNA network of mRNA targets and binding sites might not be unique, as several other viral miRNAs share seed-sequence identity with cellular miRNAs^{10,11}. This suggests that the use of host miRNA regulatory networks might be a common evolutionary strategy of multiple viruses. Although it remains to be definitively shown, the observation that exogenous expression of miR-k12-11 alters host transcripts that are involved in the innate immune response and in apoptosis suggests that KSHV encodes at least one viral ncRNA to block host antiviral defences.

Autoregulation of viral gene expression by miRNAs. In addition to regulating host gene expression, several viral miRNAs have been described that regulate viral transcripts. Simian virus 40 (SV40) is a member of the polyomavirus family, which is comprised of small (~5 kb) DNA viruses that generally form harmless, persistent infections. However, in immunocompromised hosts infection is associated with various diseases, including nephropathy, neural demyelination and tumorigenesis. SV40 expresses a precursor miRNA (pre-miRNA) late in

infection that is processed into two miRNAs that bind with perfect complementarity to the early RNAs, thereby directing their cleavage¹³. Some protein products of the early RNAs are particularly immunogenic and are recognized by components of the adaptive immune response, thus generating a strong cytotoxic T cell (CTL) response during infection (BOX 2). When *in vitro* infected cells are co-cultured with CTLs, more killing is observed in cells infected with a mutant virus that is unable to make the miRNAs. This suggests a possible role for the SV40 pre-miRNA in evading the adaptive immune response *in vivo*. Other viruses, including HCMV and Epstein–Barr virus (EBV), a lymphotropic herpesvirus that is associated with several malignancies, have been shown to encode miRNAs that downregulate expression of viral genes involved in signalling and replication^{14–16} (TABLE 1). Thus, it is possible that multiple viruses can regulate their exposure to the adaptive immune response, either directly by reducing antigen levels or indirectly by dampening viral replication. The advantage of this regulatory strategy for the virus might be that relatively little genomic space is required to autoregulate viral mRNA and/or protein expression levels.

Viral lncRNAs block host defences

Viral lncRNAs have been found in divergent families of DNA viruses, including herpesviruses and adenoviruses. Despite being among the most abundant transcripts expressed during infection (often

10⁶ or more copies per cell), a functional understanding of most lncRNAs remains incomplete or unknown (TABLE 1). One of the best-characterized viral lncRNAs is the adenovirus-associated (VA) RNA. Adenoviruses have medium-sized (~33 kb), double-stranded genomes and are associated with respiratory illness. Most serotypes of adenovirus encode two VA RNA orthologues; each of these is a ~165 nucleotide RNA polymerase III-derived RNA that folds into a conserved multi-pronged hairpin structure. VA RNAs perform the essential function of blocking the activity of cellular protein kinase R (PKR), an interferon (IFN)-induced cellular antiviral kinase (reviewed in REF. 17). EBV encodes two abundant lncRNAs called EBV-encoded RNAs (EBERs). The EBERs loosely resemble the VA RNAs in size and structure. However, unlike the VA RNAs, EBERs are strictly nuclear localized, thereby ruling out identical functions in inhibiting cytoplasmic PKR¹⁸. Interestingly, although the mechanism is unknown, EBERs can prevent IFN-mediated apoptosis in some cell types¹⁹. Thus, VA RNAs and EBERs are two viral lncRNAs that have established the precedence of viral ncRNA inactivation of host defences (FIG. 1).

Recently, the function of a third viral lncRNA has been determined. HCMV encodes β2.7, a non-coding RNA that accounts for a large fraction (~20%) of the transcripts that are expressed during lytic infection. Reeves and colleagues used a protein affinity approach, whereby they used

Table 1 | **A summary of the viral ncRNAs discussed in this paper**

Non-coding RNA	Virus	Function	Refs
Viral miRNAs			
miR-UL112	Human cytomegalovirus	Downregulates the expression of host protein MICB, thereby reducing killing by NK cells; downregulates expression of viral transcript IE72 and possibly others involved in viral replication	8,14
miR-K12-11	Kaposi's sarcoma herpesvirus	Exogenous expression shown to downregulate numerous host transcripts with putative functions, including innate immunity and apoptosis	10,11
miR-BART2	Epstein–Barr virus	Cleaves the BALF5 viral transcript, which encodes viral polymerase	5
miR-BART16, miR-BART17-5p & miRBART1p	Epstein–Barr virus	Downregulates the translation of Epstein–Barr virus LMP1, a membrane signalling protein	15
miR-S1	Simian virus 40	Cleaves early transcripts during a late stage of infection, thereby reducing T antigen protein levels	13
Viral lncRNAs			
VAI & VAIL	Adenovirus	Inhibits PKR-mediated translation inhibition	17
EBER1 & EBER2	Epstein–Barr virus	Inhibits interferon induced apoptosis	19
β2.7	Human cytomegalovirus	Binds to mitochondrial enzyme complex 1, maintains sufficient cellular ATP levels during infection, might prevent apoptosis that is due to metabolic stress	20
HSUR1 & HSUR2	Herpes saimiri virus	Possibly functions in the activation of T cells	30

EBER, Epstein–Barr virus-encoded RNA; HSUR, herpes saimiri virus U RNA; LMP1, latent membrane protein 1; lncRNA, long non-coding RNA; MICB, major histocompatibility complex class I polypeptide-related sequence B; miRNA, microRNA; NK, natural killer; PKR, protein kinase R.

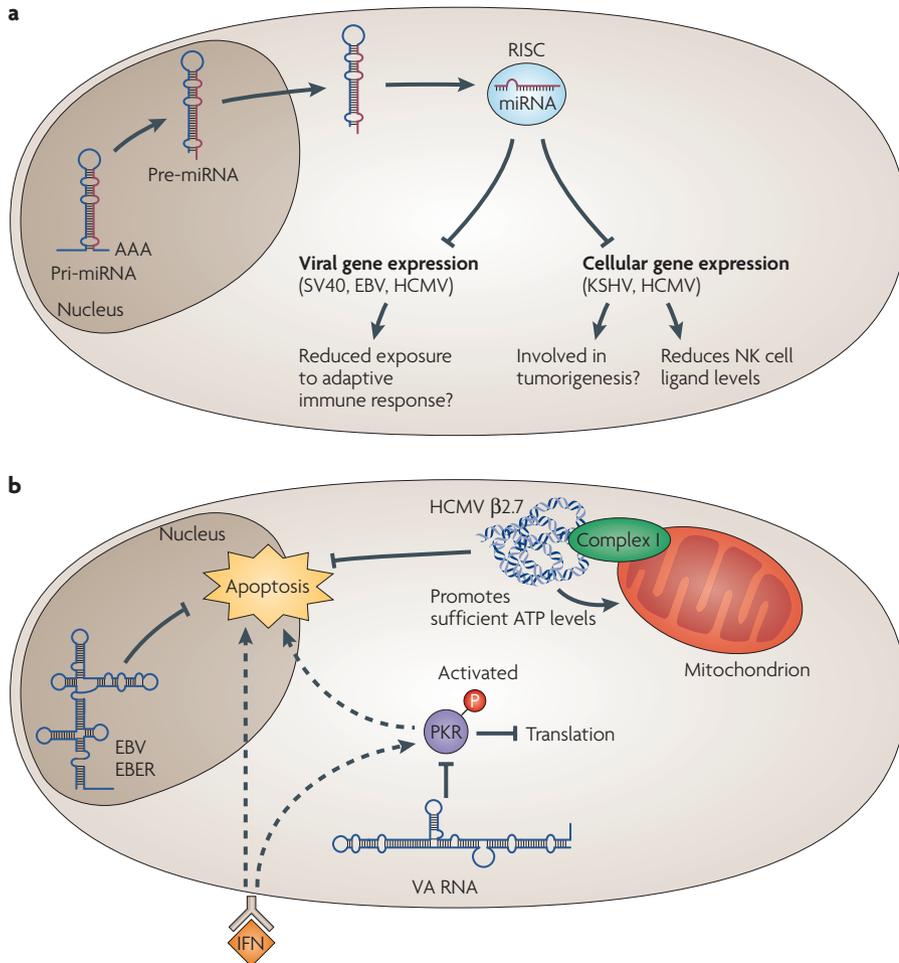


Figure 1 | Functions of viral non-coding RNAs in evading host defences. **a** | Viral microRNAs (miRNAs) target host or viral transcripts. Most viral miRNAs are generated by the nuclear and cytoplasmic cellular miRNA-processing machinery. A series of host endonucleases process the primary transcripts (pri-miRNAs) into the ~70 nucleotide precursor hairpin (pre-miRNA) and eventually the mature ~22 nucleotide miRNA. When bound by the multi-protein RNA induced silencing complex (RISC), miRNAs typically recognize target mRNAs with imperfect complementarity and thereby prevent translation. In addition, a miRNA can have perfect complementarity to an mRNA target, which results in reduced protein expression by specific cleavage of the target mRNA transcript. Simian virus 40 (SV40), Epstein-Barr virus (EBV) and human cytomegalovirus (HCMV) all encode miRNAs that downregulate viral gene expression, perhaps to promote reduced exposure to the adaptive immune response. Kaposi's sarcoma herpesvirus (KSHV) and HCMV have both been shown to encode miRNAs that regulate cellular gene expression. **b** | Viral long non-coding RNAs (lncRNAs). Viral lncRNAs are RNA polymerase II (pol II) or pol III-derived transcripts that are expressed at high abundance during infection with some DNA viruses. Two of the better-studied viral ncRNAs are the adenovirus associated (VA) RNAs and the EBV-encoded RNAs (EBERs). Some cues of viral infection promote autophosphorylation of protein kinase R (PKR), the activation of which results in the inhibition of protein translation and sometimes leads to apoptosis. VA RNAs function in the cytoplasm to block the function of activated PKR. EBERs are strictly nuclear-localized and prevent interferon (IFN)-mediated apoptosis by an unknown mechanism. The HCMV RNA β 2.7 binds to mitochondrial complex I and prevents apoptosis that is induced by mitochondrial stress, thus maintaining sufficient ATP levels for infection. NK, natural killer.

a radiolabelled fragment of β 2.7 RNA as a probe in a northwestern assay²⁰. One of the proteins identified in this screen is a component of mitochondrial complex I. Complex I is the first enzyme in the respiratory electron transport chain and has a crucial role in regulating metabolic cellular ATP levels. Complex I increases transmembrane proton potential

by oxidizing NADH, which provides an energy source for ATP synthesis in the oxidative phosphorylation electron transfer chain. Expression of β 2.7 prevented alteration of the subcellular localization of components of complex I in the presence of artificial apoptotic stimuli. Furthermore, an HCMV virus that was mutant for β 2.7 expression

was defective for growth on glucose-depleted media. Combined, these results suggest β 2.7 might help to maintain sufficient ATP levels during infection and prevent apoptosis that is associated with viral-induced metabolic stress. Thus, a common strategy becomes apparent in which viral lncRNAs are expressed at high levels to interact stoichiometrically with cellular factors to prevent cellular responses that would be damaging to productive infection.

Connections between lncRNA and miRNAs. Interestingly, some lncRNAs can give rise to miRNAs. Marek's disease virus (MDV) is an avian oncogenic virus that is similar to other alpha herpesviruses (such as herpes simplex viruses (HSVs)) and that encodes abundant non-coding latency-associated transcripts (LATs) during latent infections. In MDV, several viral miRNAs map within the LATs, suggesting they could mainly function as primary miRNAs (pri-miRNAs)²¹. Although there is little sequence similarity between the MDV and HSV LATs, it is possible that both could have a similar function as pri-miRNAs, thereby providing a satisfactory function for the enigmatic LATs. Interestingly, new activities of the VA RNAs have been reported that suggest a small fraction of the VA RNAs is processed into functioning miRNAs. The VA RNAs are so abundant during infection that even though only ~1% are processed into miRNAs, this still allows for 10⁶ VA-derived miRNAs per cell, which is on a par with the most abundant host miRNAs^{22,23}. Expression of the VA protein VA1 during infection interferes with the nuclear export of pre-miRNAs and, furthermore, inhibits the function of Dicer (a ribonuclease in the RNase III family) in the cytoplasm²². The net result is cells that exhibit hampered processing of host miRNAs and that have a large portion of RNA-induced silencing complex (RISC)-containing VA-derived miRNAs. The functional relevance of these observations and their relation to the anti-IFN activities of VA RNA remains to be shown. However, what is clear is that some virological observations that pre-date the discovery of miRNAs might now be explained by them.

What next?

In the future, viruses might serve as models to study the functions of new classes of ncRNAs. For example, several viruses, including those of the polyoma, papilloma and herpes families, encode antisense ncRNAs²⁴⁻²⁸. Recent studies have implicated antisense RNAs as having a role in directing heterochromatin formation in mammals²⁹.

Thus, it is possible that overlapping transcripts, which were previously thought of as an accidental consequence of having a compact genome, might serve to regulate the chromatin state of viral episomes. In addition, viruses might serve as sources of discovery for new classes of ncRNAs that are also encoded by the host. That lncRNAs and miRNAs from distant viral families function to inhibit antiviral cellular responses suggests some of the numerous remaining viral ncRNAs could do the same. For the viral lncRNAs, progress in understanding their function has been slow, but recent successes using cDNA expression microarray analysis and protein affinity studies might provide a formula for uncovering the functions of other RNAs^{20,30}. Finally, it is likely that the number of viral miRNAs with known mRNA targets will grow substantially in the near future. This will provide a valuable starting point to direct future functional studies. Given the early successes in developing antisense inhibitors of miRNA function, viral miRNAs might prove to be good therapeutic targets — particularly if inactivating miRNA function leads to an increased immune response. Thus, ncRNAs have set the stage for a windfall of new insights into how viruses interact with and manipulate their hosts.

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FURTHER INFORMATION

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