



# Interaction of viruses with the mammalian RNA interference pathway

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## Abstract

It has been known for some time that plants and insects use RNA interference (RNAi) as nucleic acid-based immunity against viral infections. However, it was unknown whether mammalian cells employ the RNA interference pathway as an antiviral mechanism as well. Over the past years, it has become clear that a variety of viruses, first in plants but recently in insect and mammalian viruses as well, encode suppressors of the RNAi pathway arguing for an antiviral role of this machinery. More recent findings have revealed that certain viruses encode their own microRNAs or microRNA-like RNA molecules, which are processed by the mammalian RNAi machinery. Furthermore, host-encoded microRNAs have been shown to both silence and enhance intracellular levels of viral RNAs. These findings argue that interactions between the RNAi pathway and viral genomes can profoundly affect the outcomes of the viral life cycles and contribute to the pathogenic signatures of the infectious agents.  
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## Contents

The RNA interference (RNAi) pathway . . . . .	151
Biogenesis and target recognition of microRNAs . . . . .	152
Plant and insect viruses encode suppressors of RNA silencing . . . . .	153
Inhibition of the RNA silencing pathway in invertebrate cells by animal viruses . . . . .	153
Inhibition of the RNA silencing pathway in mammalian cells by animal viruses . . . . .	153
Identification of virus-encoded microRNAs . . . . .	154
Subversion of a tissue-specific microRNA to aid in viral gene expression . . . . .	154
Defense of a retrovirus against attack by a microRNA in mammalian cells . . . . .	155
Concluding remarks . . . . .	155
Acknowledgments . . . . .	156
References . . . . .	156

## The RNA interference (RNAi) pathway

Effects of small RNAs on gene expression were first observed in 1990, when plant biologists attempted to make purple petunias more vividly purple by overexpressing the purple pigment-synthesizing enzyme, chalcone synthase. Surprisingly, the transgene-carrying plants turned white, indicating that the endogenous gene and the transgene were co-

suppressed (Napoli et al., 1990; van der Krol et al., 1990). Subsequently, this posttranscriptional gene silencing (PTGS) was attributed to the generation and action of small, double-stranded RNAs (Hamilton and Baulcombe, 1999). Since then, gene silencing by small duplex RNA molecules has been termed “RNA interference” or RNAi (Rocheleau et al., 1997). Biochemical analysis of the small interfering RNAs (siRNAs) revealed that they were processed from larger double-stranded precursors (Hammond et al., 2000; Zamore et al., 2000) by an ATP-dependent RNase III-like nuclease termed Dicer (Bernstein et al., 2001; Nykanen et al., 2001). In mammalian cells,

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the processed double-stranded siRNAs are approximately 21 nucleotides in length, contain a monophosphate at their 5' ends and two unpaired nucleotides at their 3' ends (Elbashir et al., 2001a, 2001b). After processing by Dicer, the siRNAs are then transferred to the RNA-induced silencing complex (RISC) which guides the siRNAs to their target RNAs (Hammond et al., 2000; Zamore et al., 2000). Perfect base complementarity between an siRNA and its target mRNA usually results in the degradation of the mRNA. Today, tailored duplex RNAs are widely used to reduce the expression of target genes in eukaryotic cells.

In parallel, it was observed that double-stranded RNA molecules could specifically interfere with gene expression in the nematode *C. elegans* (Fire et al., 1998). Subsequent genetic screens identified small temporal RNA (stRNA) molecules that were similar in size to the siRNAs. In contrast to the siRNAs, however, these molecules were single-stranded and exhibited pairing with genetically defined target mRNA sequences which were only partially complementary to the stRNA (Bartel, 2004). Specifically, stRNAs lin-4 and let-7 were found to interact with the 3' noncoding regions of target lin-14 and lin-41 mRNAs, respectively, causing reduced accumulation of the mRNA-encoded proteins. These findings prompted several

investigators to search for stRNA-like molecules in a variety of organisms, leading to the discovery of hundreds of highly conserved RNA molecules with stRNA-like structural properties (Lewis et al., 2003). These small RNAs are now known as microRNAs (miRNAs). They are processed from hairpin-like precursor molecules by Dicer and are present in virtually every tissue of every animal investigated (Lagos-Quintana et al., 2002). Thus, the RNAi pathway guides two distinct RNA species, double-stranded siRNAs and single-stranded microRNAs, to the cytoplasmic RISC complex which presents them to their target molecules.

### Biogenesis and target recognition of microRNAs

In general, microRNAs are transcribed by polymerase II as large precursor RNAs which are processed by the nuclear ribonuclease III-like enzyme Drosha aided by protein DGCR8 (Tomari and Zamore, 2005). The processed pre-microRNA molecules, approximately 70 nucleotides in length, are then exported via the Exportin 5 receptor to the cytoplasm (Yi et al., 2003) where they are further processed by Dicer, followed by asymmetric assembly of one of the microRNA strands into the RISC complex (Tomari et al., 2005) (Fig. 1).

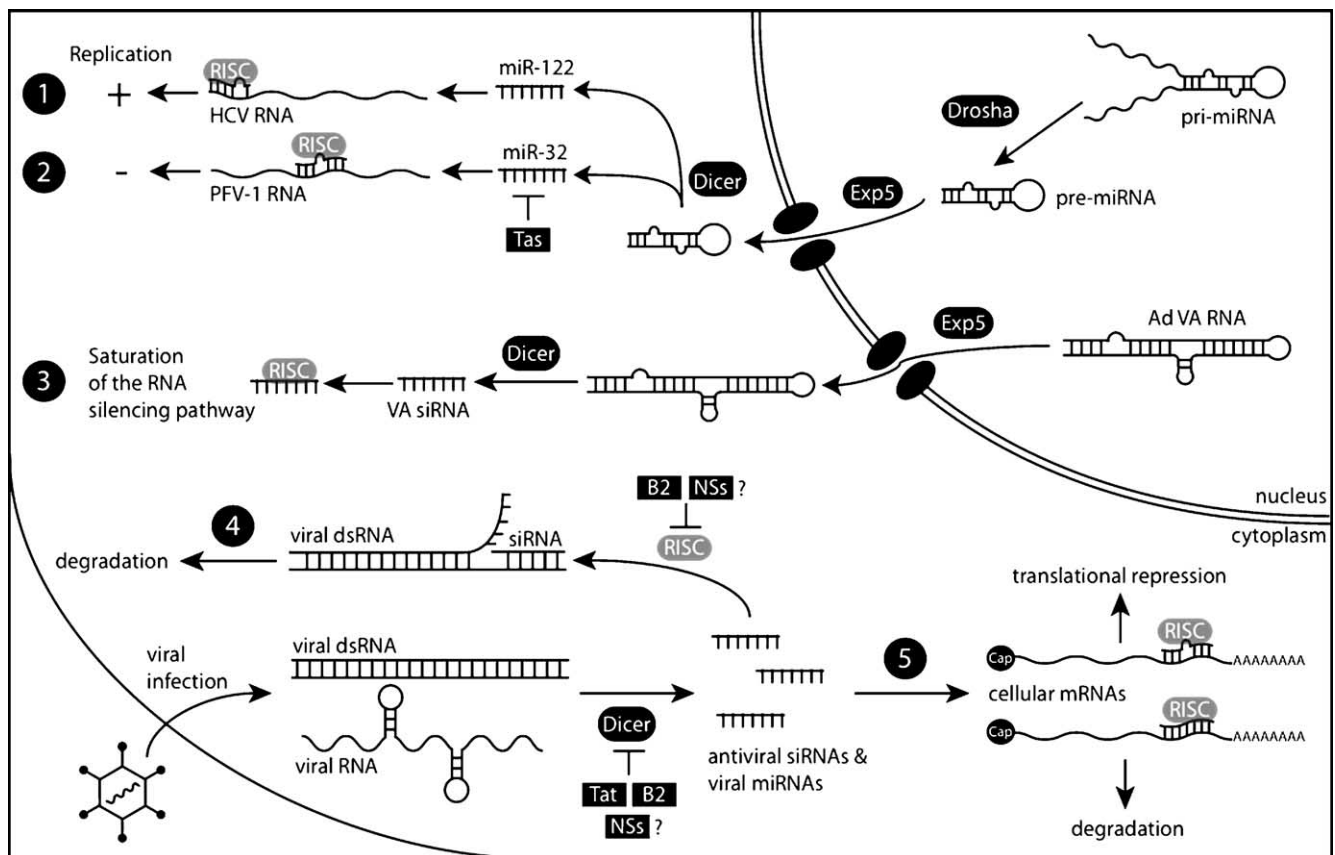


Fig. 1. Interactions of viruses with the RNA interference machinery. (1) Liver-specific microRNA miR-122 binds to the 5' noncoding region of HCV RNA and promotes viral replication. (2) MiR-32 binds to PFV-1 RNA and lowers viral RNA amounts. The viral protein Tas inhibits microRNA activity. (3) Adenovirus produces large amounts of VA RNAs, that are transported via Exportin 5 (Exp5), processed by Dicer and incorporated into RISC, resulting in the saturation of the entire RNAi pathway. (4) Viral dsRNA induces the production of viral siRNAs by Dicer, resulting in the degradation of viral RNAs. HIV-1 Tat, NoV B2 and LACV NSs counteract this defense. (5) Viruses such as EBV, KHSV and SV40 encode their own microRNAs that are used to modulate host gene expression or viral expression. See text for details.

How does a specific microRNA recognize its target? Recent findings have shown that the 5' 7–8 nucleotides of a microRNA engages in perfect Watson–Crick base pairing with its target mRNA, leading to the formation of an A-form RNA duplex (Lewis et al., 2005). While there has been wide spread speculation that microRNAs inhibit the translation of mRNAs in microRNA/mRNA complexes (Doench and Sharp, 2004) (Olsen and Ambros, 1999; Pillai et al., 2005), very recent data have shown that interaction of microRNAs with natural target mRNAs can also lead to mRNA degradation (Bagga et al., 2005). Therefore, it is likely that the microRNP or RISC complex can induce degradation, translational repression, or both, of target mRNAs. The choice of mechanism may be dependent on the number of microRNA-binding sites and the degree of base complementarity between the microRNA and the target mRNA.

### **Plant and insect viruses encode suppressors of RNA silencing**

Interactions of viruses with the RNAi machinery were first noted during infections of plants and insects. Specifically, it was observed that the infected hosts used RNA silencing as a nucleic acid-based immunity to destroy viral RNA. To counteract this defense, several viruses have developed a wide variety of suppressors of RNA silencing (SRS). Two well-studied examples of plant SRS proteins are tombusviral P19 and potyviral HcPro proteins. Whereas P19 specifically binds siRNAs, inhibiting their incorporation into the RISC complex (Lakatos et al., 2004), HcPro prevents the microRNA-directed cleavage of target RNAs and is believed to function at a step that involves Dicer and RISC (Kasschau et al., 2003). Detailed descriptions of plant SRS proteins can be found in several excellent publications (Roth et al., 2004; Silhavy and Burgyn, 2004; Vance and Vaucheret, 2001; Voinnet, 2001, 2005). Curiously, several plant viral SRS proteins from a wide range of viruses can enhance the replication of heterologous viruses, suggesting that common components of the RNAi machinery are targeted by the SRS proteins (Dunoyer et al., 2004; Kasschau et al., 2003; Pruss et al., 1997).

### **Inhibition of the RNA silencing pathway in invertebrate cells by animal viruses**

The next milestone in the interaction of the RNA silencing machinery and viruses was set by Ding and colleagues with the discovery that Flock house virus (FHV) infection of *Drosophila* cells first induced and subsequently suppressed a host-induced antiviral RNA silencing response (Li et al., 2002). Subsequently, the viral B2 protein was found to inhibit RNA silencing in both insect cells and in transgenic plants, demonstrating that the SRS proteins are functioning across kingdoms (Li et al., 2002, 2004). Nodamura virus (NoV), which can infect both insects and mammals, encodes a B2 protein that is very similar to the FHV B2 protein. Recently, it was found that the NoV B2 protein can inhibit the mammalian RNAi pathway by binding to pre-Dicer substrates and mature

siRNA molecules, resulting in interference with Dicer-directed cleavage and, probably, incorporation of siRNAs into the RISC complex (Sullivan and Ganem, 2005) (Fig. 1). Similarly, the tripartite negative-stranded La Crosse virus, a bunyavirus which can infect both insect and mammalian hosts, was found to encode a nonstructural protein, NSs, which displays RNA silencing suppressor activity in human 293T cells (Soldan et al., 2005) (Fig. 1). Another bunyavirus, Tomato spotted wilt virus, subsequently was found to encode an NSs protein with similar activity (Bucher et al., 2003; Takeda et al., 2002). It is as yet unknown whether either bunyaviral NSs protein functions as an SRS in insect cells. Overall, these findings demonstrate that viruses can interfere with the RNAi pathway by encoding suppressor proteins that act across kingdoms. Due to the wide host range of the viruses and the conservation of the RNAi machinery, however, it is not clear in some cases which host the virus has evolved to combat.

### **Inhibition of the RNA silencing pathway in mammalian cells by animal viruses**

In contrast to the examples from plant and invertebrate viruses, which encode SRS proteins to inhibit the degradation of viral RNA by the RNAi pathway (Voinnet et al., 1999), it was thought that mammalian viruses might not need to interfere with the RNAi pathway because of the ubiquity and effectiveness of the interferon system which responds to the accumulation of viral double-stranded RNAs by inducing the synthesis of a large group of genes which exert inhibitory effects on viral gene expression (reviewed in (Gale et al., 2000; Katze et al., 2002). For example, protein kinase PKR, when activated by cytoplasmic double-stranded RNAs, leads to the rapid phosphorylation of eukaryotic initiation factor eIF2 and subsequent inhibition of both host and viral mRNA translation (reviewed in Jagus et al., 1999; Kaufman, 1999). However, with the discovery by Elbashir and colleagues that the expression of small siRNAs does not induce the mammalian interferon machinery (Elbashir et al., 2001a, 2001b), the question arose whether mammalian viruses could be targeted by the generation of small siRNAs by the RNAi machinery in mammalian cells, and whether viruses would have means to avoid such an attack.

The first answers to these questions came from studies with adenoviruses. It has been known for a long time that the adenoviral genome encodes VA (virus-associated) RNAs which are expressed by host RNA polymerase III. VA RNAs are highly structured RNAs, approximately 160 nucleotides in length, which can accumulate to  $10^8$  molecules per infected cell (Mathews and Shenk, 1991). VA1 RNA was shown to bind to PKR, thereby inhibiting its activation by viral double-stranded RNAs and thus allowing translation of viral mRNAs (reviewed in (Mathews and Shenk, 1991). Subsequently, it was discovered that VA1 RNAs are exported from the nucleus to the cytoplasm by the Exportin 5 factor (Gwizdek et al., 2003), the same export receptor that exports pre-microRNAs (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003) (Fig. 1). Recent data have shown that VA1 RNAs can inhibit nuclear-cytoplasmic translocation of pre-microRNAs by competing

for the Exportin 5-dependent nuclear export (Lu and Cullen, 2004). In addition, it has been reported that both VA1 and VA2 RNAs competitively suppress DICER activity (Fig. 1). DICER can process bound VA RNAs leading to the generation of siRNAs that are associated with RISC complexes during lytic infection (Andersson et al., 2005). Natural cellular or viral targets for VA-siRNAs are yet unknown. It is therefore likely that VA RNAs aid in viral subversion of infected hosts by preventing activation of PKR as well as by inhibiting several steps of the host RNAi pathway (Fig. 1).

These findings raised the question whether other virus-encoded proteins that inactivate PKR also inhibit the host RNAi pathway. Indeed, influenza virus NS1 and vaccinia virus E3L proteins, which are both inhibitors of PKR (Garcia-Sastre, 2002), were the first discovered proteins encoded by mammalian viruses shown to be active against the invertebrate RNAi machinery (Li et al., 2004). The ability of these proteins to suppress the RNAi pathway was shown in *Drosophila* cells and is believed to be mediated by the dsRNA-binding domains located in both proteins (Li et al., 2004). Because a functional PKR pathway has not been detected in insect cells, the inhibitory effects of NS1 and E3L in *Drosophila* cells are most likely due to inhibition of the RNAi pathway. Whether these two viral proteins suppress RNA silencing in infected mammalian cells remains to be shown.

### Identification of virus-encoded microRNAs

Studies with VA RNAs have raised the question whether siRNAs generated from viral sequences can function as microRNAs, modulating either viral or host cell gene expression. To address this possibility, Pfeffer et al. (2004) isolated total small RNAs from a Burkitt's lymphoma cell line that was latently infected with Epstein–Barr virus (EBV) (Fig. 1). Cloning and sequencing of these RNA species revealed that approximately 4% of the recovered cDNAs were viral sequences. Five of these RNAs were encoded in two regions of the EBV genome, and were predicted to reside in the stem–loop structures characteristic of microRNA precursor genes. One of these viral microRNA species, miR-BART2, was predicted to be perfectly complementary to the viral DNA polymerase BALF5 gene. If functional, targeted mRNA cleavage should result in the appearance of a processed 3.7 kb RNA species of the full-length 5.0 kb BALF5 mRNA, generated by a miR-BART2-induced siRNA-type mechanism (Pfeffer et al., 2004). Indeed, a 3.7-kb BALF5 mRNA species can be detected in EBV-replicating cells (Fumari et al., 1993). Several cellular targets of EBV-encoded microRNAs have been predicted, but experimental verification of any functional interaction has not yet been reported. Using improved computational algorithms, microRNA-encoding genes were subsequently predicted and their expression biochemically verified in other members of the herpesvirus family (Fig. 1). Specifically, human cytomegalovirus expresses at least 5 microRNAs (Grey et al., 2005; Pfeffer et al., 2005), which are generally expressed at early times during viral infection (Grey et al., 2005). In addition, Kaposi's sarcoma-associated

herpesvirus (KSHV), human herpesvirus 8, expresses 11 microRNAs in latently infected cells (Cai et al., 2005; Pfeffer et al., 2005; Samols et al., 2005). Curiously, 10 of the 11 KSHV microRNAs are predicted to be expressed from a single primary microRNA transcript, which resides in a single intron. While cellular targets for the KSHV microRNAs might be predicted to be down-regulated during KSHV infection (Cai et al., 2005), no genetic or biochemical evidence for KSHV microRNA-target mRNA interactions has been reported yet. Interestingly, the algorithms that predicted verifiable microRNAs from herpes virus genomes predicted no microRNAs encoded in, for example, the human immunodeficiency virus (HIV) genome (Pfeffer et al., 2005). However, two recent studies have reported that the host RNAi pathway can generate viral siRNAs from *env*- and *nef*-encoding regions of the HIV genome (Bennasser et al., 2004, 2005; Omoto and Fujii, 2005; Omoto et al., 2004); intriguingly, the HIV Tat protein seems to function as an SRS by altering the ability of Dicer to process helical precursor RNAs into siRNAs (Bennasser et al., 2005). It is possible that the generation of HIV-derived microRNAs is dependent on the experimental system or the intracellular concentrations of substrate RNAs. As yet, it is unknown whether HIV-derived microRNAs or siRNAs are produced during natural HIV infections.

The only virus-encoded microRNAs known to have functional roles in the infectious cycle are microRNAs processed from late viral transcripts in simian virus 40 (SV40)-infected cells (Sullivan et al., 2005). Similarly to the herpesvirus studies, Sullivan and colleagues used a computer-aided algorithm to identify microRNAs encoded in the SV40 genome. Subsequent Northern blot analysis verified the presence of microRNAs processed from late SV40 transcripts (Fig. 1). The SV40 microRNAs were excised from both arms of a predicted pre-microRNA hairpin structure and found both to be perfectly complementary to early SV40 transcripts that encode both the large “T” and small “t” SV40 tumor antigens. As predicted, these target mRNAs were cleaved in wildtype SV40 infections, but not in infections with mutant SV40 that contained mutations to disrupt the predicted pre-microRNA hairpin structure. As a result, the abundance of both large and small tumor antigens was reduced at late times in wildtype, but not in mutant SV40-infected cells. Surprisingly, downregulation of the tumor antigens did not affect the final viral yield in cultured cells, pointing to a role for SV40 microRNAs in a step that did not include viral gene expression. Indeed, it was found that SV40 microRNA-mediated downregulation of the tumor antigens resulted in reduced susceptibility to and activation of responsive cytotoxic T lymphocytes (Sullivan et al., 2005). Thus, SV40 may subvert the host RNAi machinery to process virus-encoded microRNAs whose activities lead to virus escape from antiviral attacks exerted by the host immune response.

### Subversion of a tissue-specific microRNA to aid in viral gene expression

Very recently, an example emerged whereby a viral genome subverts a host microRNA to increase viral gene expression.



Specifically, Jopling and colleagues discovered that a liver-specific microRNA, miR-122, upregulates the expression of the hepatitis C virus (HCV) genome in cultured liver cells; sequestration of miR-122 by methylated oligonucleotides resulted in loss of HCV RNA (Jopling et al., 2005) (Fig. 1). Subsequently, the binding site for miR-122 was mapped to the very 5' end of the viral genome. This was curious, because no animal microRNAs (Lewis et al., 2005), and only two plant microRNAs (Sunkar and Zhu, 2004), have been shown to interact with the 5' noncoding regions of their target mRNAs. Mutations in the miR-122 binding site at the 5' end of the HCV genome abolished viral RNA accumulation, which could, however, be restored by ectopic expression of miR-122 molecules that contained mutations to restore predicted base complementarity. Studies with replication-defective genomes revealed that miR-122–HCV interactions affected primarily viral RNA replication and not mRNA translation or RNA turnover. These findings provide an unprecedented example of a virus exploiting a cellular microRNA for its own purposes (Jopling et al., 2005), raising many questions. Does any microRNA targeted to the HCV genome regulate viral genome amplification? Curiously, HCV RNA can replicate in non-hepatic cells (Zhu et al., 2003) raising the question of whether non-hepatic microRNAs regulate viral RNA amplification in these cells or whether the role of miR-122 in regulating HCV gene expression occurs only in the liver. Does the microRNA help to anchor the membrane-bound viral replication complex? What are the natural targets for miR-122 in the liver and is target recognition altered when HCV RNA concentration is high in this tissue? Finally, pegylated interferon- $\alpha$  and ribavirin therapy against HCV is frequently ineffective, particularly in patients infected with genotype 1; thus, there is a need to search for alternative antiviral targets (De Francesco and Migliaccio, 2005). Sequestration of miR-122 could provide a possible antiviral tool against a rapidly evolving viral genome.

#### Defense of a retrovirus against attack by a microRNA in mammalian cells

In contrast to HCV, a retrovirus, primate foamy virus-1 (PFV-1), seems to have evolved an SRS function to counteract the function of a host microRNA in mammalian cells (Lecellier et

al., 2005). In particular, it was found that microRNA miR-32 restricts the expression of PFV-1 in cultured human cells (Fig. 1). In the presence of plant viral SRS p19 (see above), PFV-1 RNA accumulation could be restored. The target for cellular miR-32 resides in PFV-1 open reading frame 2. MiR-32 binding reduced viral RNA abundance and PFV-1 RNA levels could be enhanced by introducing two point mutations into the miR-32 target sequence. Subsequently, it was found that PFV-1 encoded Tas protein counteracts the RNAi pathway in infected cells (Lecellier et al., 2005) (Fig. 1), leading to a general over-expression of microRNAs, an effect that also was observed for the potyviral HcPro RNA silencing suppressor in plants (Dunoyer et al., 2004). It is intriguing to speculate why PFV-1 evolved the SRS Tas function instead of simply mutating the target sequence of miR-32 while conserving the codon information. It is possible that this region is very conserved for an unknown reason or, more probably, miR-32 is not the only component of the RNA silencing machinery that PFV-1 must counteract.

#### Concluding remarks

A picture is emerging in which mammalian viruses, like those that infect plants and invertebrates, have developed different strategies to inhibit and, in some cases, exploit the RNA silencing machinery (Fig. 1). RNA silencing is apparently used by mammals as a nucleic acid-based immunity in addition to the dsRNA-inducible PKR pathway and protein-based immunity. It is striking that most identified mammalian RNA silencing suppressors also function to inhibit the PKR pathway (Table 1). It has been hypothesized that these two pathways act in a synergistic manner in the early phases of viral infections, whereas protein-based innate immunity is active several hours after infection begins (Chen et al., 2005). Perhaps not surprisingly, certain viruses, such as SV40 and HCV, have evolved to utilize the microRNA pathway to their own advantages. One can only anticipate with excitement what will be learned about the roles of microRNAs, encoded by herpesviruses and those to be discovered in other viruses, in virus–host interactions. Considering that microRNAs are predicted to regulate a third of human genes, they are likely to play prominent roles in viral life cycles as well.

Table 1  
Suppressors of RNA silencing (SRS) and their effects on double-stranded RNA activated protein kinase PKR in mammalian cells

SRS	Virus	Viral family	Genome	Comments	PKR inhibition
NS1	Influenza A/B/C	Orthomyxovirus	ssRNA (–)	SRS function shown in insect cells	Yes
E3L	Vaccinia virus	Poxvirus	dsDNA	SRS function shown in insect cells	Yes
B2	Nodamura virus	Nodavirus	ssRNA (+)	Inhibition of Dicer-mediated cleavage by binding to dsRNA precursors	?
NSs	La Crosse virus	Bunyavirus	ssRNA (–)	Inhibition of Dicer-mediated cleavage	?
Tat	HIV-1	Retrovirus	ssRNA (+)	Inhibition of Dicer-mediated cleavage	Yes
Tas	PFV-1	Retrovirus	ssRNA (+)	Inhibition of microRNA function, causes unspecific accumulation of microRNAs	?
VA RNAs	Adenovirus	Adenovirus	dsDNA	Saturation of Exportin5, Dicer and RISC	Yes

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