PERSPECTIVE

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Is RNA interference involved in intrinsic antiviral immunity in mammals?

Bryan R Cullen

RNA interference constitutes a key component of the innate immune response to viral infection in both plants and invertebrate animals and has been postulated to have a similar protective function in mammals. This perspective reviews the available data addressing whether RNA interference forms part of the mammalian innate immune response and concludes that the popular hypothesis in favor of that possibility remains far from proven and may not be valid.

In 1998, it was demonstrated[1] that injection of long double-stranded RNA (dsRNA) into nematodes induces the post-transcriptional silencing of genes encoding homologous mRNA, a process called 'RNA interference' (RNAi). It is now apparent that the mechanisms that mediate RNAi have been evolutionarily conserved in all multicellular eukaryotes, thus indicating that this unique form of homology-dependent gene silencing is key to one or more aspects of eukaryotic biology. One obvious potential function for the RNAi machinery would be to defend cells against viruses that express dsRNA as part of their life cycle. Indeed, there is compelling evidence indicating that RNAi is critical in curtailing viral infections in both plants and invertebrates. Moreover, it can be readily demonstrated that the artificial induction of an antiviral RNAi response in mammalian cells can confer strong protection against a wide range of pathogenic viruses[2]. Nevertheless, it remains unclear whether RNAi is involved in antiviral defense in mammalian cells in physiological conditions.

'MicroRNA' and RNAi

No real understanding of RNAi in vertebrate cells is possible without an appreciation of the biogenesis and function of 'microRNA' (miRNA). These miRNAs are noncoding RNAs about 22 nucleotides in length expressed by all metazoan eukaryotes[3]. The human genome encodes over 300 different miRNA molecules that are believed to be key to the post-transcriptional regulation of many aspects of cellular differentiation.

The miRNAs are initially transcribed as part of one arm of an RNA stem-loop structure of about 80 nucleotides that in turn forms part of a longer primary miRNA (pri-miRNA) transcript[4] (**Fig. 1**). The first step in miRNA processing occurs in the nucleus and involves recognition of key elements of the secondary structure of the pri-miRNA stem-loop by the RNase III enzyme Drosha and its cofactor DGCR8 (refs. 5,6). The Drosha-DGCR8 heterodimer cleaves the pri-miRNA stem-loop about 22 nucleotides away from the junction of the stem and the terminal loop, leaving a characteristic two-nucleotide 3′ overhang. The resulting precursor miRNA (pre-miRNA) hairpin of about 60 nucleotides is then bound by the nuclear export factor exportin 5 (Exp5) acting in concert with the GTP-bound form of its cofactor Ran[7,8]. This recognition is again dependent on RNA structure and optimally requires an RNA stem of 16 base pairs or more flanked by a short, approximately two-nucleotide 3′ overhang[9]. Bound pre-miRNA is transported to the cytoplasm, where hydrolysis of the GTP moiety induces its release.

Cytoplasmic pre-miRNA is recognized by a third heterodimer, consisting of the RNase III enzyme Dicer and its cofactor TRBP[10,11] (**Fig. 1**). Once again, structure is important for recognition, although the only requirements (which are not absolute) are an RNA stem of 19 base pairs or more and a two-nucleotide 3′ overhang. The Dicer-TRBP complex binds the base of the pre-miRNA hairpin and cleaves about 22 nucleotides away, leaving another two-nucleotide 3′ overhang and removing the terminal loop[12]. Dicer and TRBP then facilitate the assembly of one strand of this miRNA duplex intermediate into a protein 'effector complex' called the RNA-induced silencing complex (RISC)[13], where it acts as a 'guide RNA' to direct RISC to homologous mRNA species[14]. Binding of RISC can inhibit mRNA function by inducing cleavage of the target sequence or by inhibiting mRNA translation[3]. Cleavage of a bound mRNA by RISC requires extensive sequence homology, whereas translational inhibition can occur after binding of RISC to mRNA with only partial homology to the miRNA.

Based on the available empirical data, RNAi can be induced in vertebrate cells only by the introduction or expression of RNA that mimics one of the intermediates in the miRNA biogenesis pathway (**Fig. 1**). The first to be described were small interfering RNA (siRNA) duplexes, dsRNAs of about 19 base pairs bearing two-nucleotide 3′ overhangs that mimic miRNA duplex intermediates[15]. A second method for RNAi induction involves the expression of short hairpin RNAs (shRNAs) that function as orthologs of pre-miRNA hairpins[16]. It is also possible to construct artificial pri-miRNA transcripts that require processing by both Drosha and Dicer to give rise to siRNA–artificial miRNA[17].

A final method for inducing RNAi, which functions well in invertebrates and plants but not in somatic mammalian cells, involves the introduction or expression of long dsRNA[1,18]. This is then processed from both termini by Dicer to give siRNA duplexes that can 'program'

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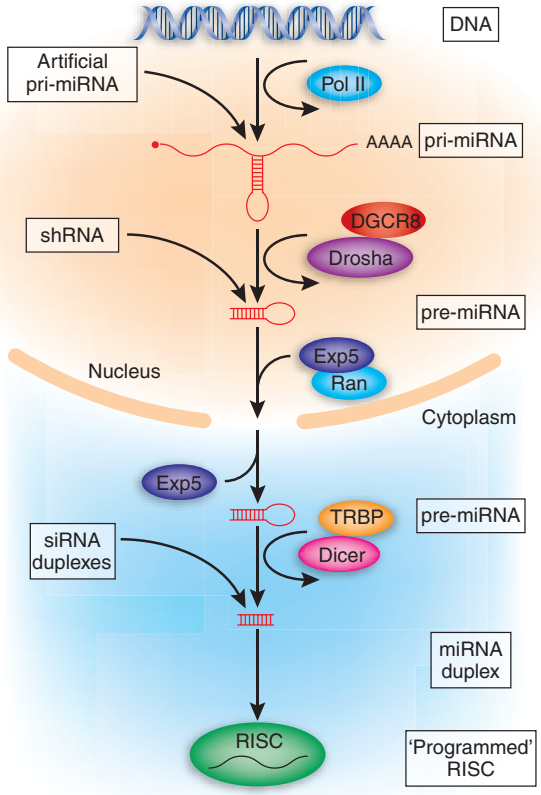

Katie Ris

Figure 1 The miRNA biogenesis pathway in vertebrate cells. 'Artificial' siRNA can be generated using this pathway if initially expressed as part of an artificial pri-miRNA, a short hairpin RNA (shRNA) or as one strand of an siRNA duplex. In addition, in invertebrates and plants, Dicer can directly process endogenously expressed or transfected long dsRNA to give rise to siRNA duplexes. Pol II, RNA polymerase II. Adapted from ref. 57.

RISC[12,19]. However, in mammalian cells, long dsRNA sequences (more than 30 base pairs in length) are potent inducers of the interferon response and its various effector molecules such as PKR, which inhibits translation, and RNase L, which degrades mRNA[20]. Given these global, relatively nonspecific responses to dsRNA, it is perhaps not unexpected that induction of RNAi by long dsRNA has not been detected in mammalian cells[18]. Indeed, it remains unclear whether the introduction of long dsRNA into mammalian somatic cells even results in the production of siRNA.

RNAi as an intrinsic antiviral defense in plants and invertebrates

All RNA viruses, except retroviruses, produce long, perfect dsRNA molecules in infected cells that represent essential intermediates in genomic RNA production. Many DNA viruses also generate large amounts of dsRNA because of convergent transcription of their small, tightly packed genomes. In contrast, although cellular RNA molecules certainly have secondary structure, most feature short, imperfect stems that are distinct from the long, perfect dsRNA molecules that are characteristic products and/or intermediates in many virus replication cycles.

Long dsRNA therefore is recognized as foreign and can trigger a range of intrinsic responses, many of which normally inhibit virus replication. If RNAi were indeed one such protective response in mammalian cells, then at least three predictions logically follow. First, viral infection should result in the production of siRNA of viral origin; second, inhibition of the RNAi response should enhance virus replication; and third, as an adaptive response to that antiviral mechanism, many viruses should have evolved gene products that specifically inhibit RNAi. All three of these criteria have been fully met in the case of virus infection in plants. Indeed, siRNA was first identified in plants undergoing RNAi in response to infection by the RNA virus potato virus X; only later was siRNA identified in invertebrate animals[21,22]. Similarly, inhibition of RNAi in plants increases their susceptibility to many plant viruses[23,24]. Finally, many data have demonstrated that almost all plant viruses encode one or more 'suppressor of RNA silencing' (SRS) proteins, which target several key steps in the RNAi response[25,26]. The expression of these diverse SRS proteins by plant viruses, which echoes the large number of inhibitors of the interferon response expressed by vertebrate viruses[20], confirms the potential importance of RNAi in controlling viral infection in plants.

It is now apparent that invertebrates, and more specifically nematodes and insects, also use RNAi to help control viral infections. Flock house virus (FHV), a member of the nodavirus family, can infect both insects and vertebrate cells. FHV infection of cultured drosophila cells results in the appearance of FHV-specific siRNAs, and wild-type FHV infection is enhanced by disruption of the cellular RNAi response[27]. Notably, whereas mutational inactivation of the FHV *B2* gene, which encodes a viral SRS that acts as an inhibitor of Dicer function, blocks FHV replication in insect cells, B2-deficient FHV can be 'rescued' by artificial inhibition of cellular RNAi responses[27]. Evidence has shown that RNAi is also important as an innate antiviral mechanism in intact insects[28–30]. Fruit flies that lack an intact *dicer-2* gene and hence are unable to process long dsRNA into siRNA, show enhanced susceptibility to infection by FHV and several other unrelated RNA viruses. Infection of *dicer-2* mutant flies also results in a much greater viral load than that of wild-type flies[28,29]. Moreover, although FHV infection of wild-type fruit flies requires the viral B2 protein, replication of B2-deficient FHV can again be 'rescued' by inactivation of the host *dicer-2* gene. Similarly, the alphavirus O'nyong-nyong virus has been found to replicate to far higher titers in mosquitoes that are unable to mount an RNAi response[29]. That is a notable finding, as it suggests that innate RNAi responses may modulate the ability of mosquitoes to act as vectors for human infection by alphaviruses such as O'nyong-nyong virus as well as other important viral pathogens, such as the flaviviruses dengue and yellow fever.

Analysis of the B2 protein of nodamura virus, a distant relative of FHV, has demonstrated that the nodamura virus B2 protein can block an antiviral RNAi response in infected mosquito cells and can inhibit artificially induced RNAi in mammalian cells[31,32]. The nodamura virus B2 protein, as well as the B2 protein encoded by a third nodavirus, greasy grouper nervous necrosis virus, also enhances the accumulation of nodavirus RNA in infected mammalian cells[31,33]. However, those studies did not address whether infection of mammalian cells with nodamura virus or greasy grouper nervous necrosis virus induces virus-specific siRNA or whether the effect of the B2 protein could be 'phenocopied' by the inhibition of RNAi, as has been shown in FHV-infected drosophila cells[27,28]. It therefore remains entirely possible that the positive effect of B2 on the infection of animal cells by nodavirus reflects another mechanism of action, such as inhibition of the interferon response.

Although little is known about natural viral infections of nematodes, *Caenorhabditis elegans* is susceptible to infection by FHV and the mammalian virus vesicular stomatitis virus. Vesicular stomatitis virus–derived siRNA can be readily detected in infected nematodes, and vesicular stomatitis virus replication is enhanced in nematodes lacking components of the RNAi machinery[34,35]. Similarly, FHV infection of *C. elegans* induces a potent antiviral RNAi response capable of blocking

the replication of FHV variants lacking the viral B2 protein, a viral SRS[36]. These data collectively indicate that RNAi probably forms a key part of the innate immune response to viral infection in a wide range of invertebrate species.

Is RNAi important for fighting viral infection in mammals?

As noted above, artificially induced RNAi responses in mammalian cells can confer protection against a wide variety of pathogenic viruses[2]. Such results naturally raise the following question: Do mammalian cells actually mount a protective RNAi response after viral infection? At present there is no published evidence addressing whether inhibition of the RNAi response can enhance virus replication in mammalian cells. However, there have been efforts to identify siRNA in virus-infected human cells. The most complete studies, by Pfeffer *et al.*[37], have analyzed small RNAs expressed in cells infected by a wide range of viruses, including the DNA viruses human cytomegalovirus, Kaposi sarcoma–associated herpesvirus, Epstein-Barr virus and mouse herpes virus 68, as well as the retrovirus human immunodeficiency virus type 1 (HIV-1) and the RNA viruses yellow fever virus and hepatitis C virus. That report fails to identify any viral siRNA but does identify several virally encoded miRNA molecules in DNA virus–infected cells, which clearly suggests that these viruses are in fact using the cellular RNAi machinery for their own ends[37]. These authors also show that hepatitis C virus infection does not inhibit the induction of an artificially induced RNAi response directed at a cellular gene. These data collectively indicate that RNAi responses are not induced in response to infection of human cells by a range of pathogenic viruses, including two RNA viruses, yellow fever virus and hepatitis C virus, which generate long dsRNA during their life cycle.

In direct contradiction to the result discussed above, Bennasser *et al.*[38] have reported the existence of a single siRNA in HIV-1-infected cells. The siRNA 'target sequence' maps to the viral Rev response element (RRE), a highly structured RNA element that facilitates the nuclear export of HIV-1 mRNA[39,40]. These investigators also argue that the proposed viral siRNA could inhibit HIV-1 replication. However, there are several problems with the data presented in that study[38] that collectively indicate the likelihood that, at least in part, the conclusions made are incorrect.

Although Bennasser *et al.*[38] propose that the purported HIV-1 siRNA derives from a perfect 19–base pair RNA stem (**Fig. 2a**), extensive analysis of the structure of the RRE, both *in vitro* and *in vivo*[40,41] has shown that these sequences do not in fact form base pairs (**Fig. 2b**). And even if these RRE sequences were to form base pairs, published data have demonstrated that Dicer cleaves short dsRNA stems very inefficiently when they are flanked by unstructured RNA sequences (which would be true for the HIV-1 RRE)[42]. Although Bennasser *et al.*[38] present data that could be interpreted as showing that Dicer can excise this candidate HIV-1 siRNA *in vitro*, the artificial RNA substrate used in their analysis was designed to contain two complementary 19–base pair sequences flanked by a two-nucleotide 3′ overhang and linked by a short terminal loop: a perfect Dicer substrate similar in structure to a pre-miRNA (**Figs. 2a** and **3a**). However, such an artificial substrate contrasts sharply with what is found in natural HIV-1 RRE RNA, in which the two 19-nucleotide sequences do not form base pairs (**Fig. 2b**), are flanked by several thousand nucleotides of largely unstructured RNA and are separated by 197 nucleotides.

It is well established that even 'weak' RNA secondary structures can block the access of RISC to a potential mRNA target[43]. Indeed, experiments analyzing HIV-1 variants selected in culture for resistance to an artificial siRNA have demonstrated that a point mutation stabilizing an RNA structure involving the siRNA target protects HIV-1 against RNAi mediated by that siRNA[44]. As the putative siRNA described by Bennasser *et al.*[38] is complementary to part of a highly stable RNA structure that has been fully confirmed *in vitro* and *in vivo*[39–41] (**Fig. 2b**), it is not expected that this siRNA would have any effect.

It is worth noting that Bennasser *et al.*[38] do present data showing that HIV-1 infection results in the appearance of RNA about 21–24 nucleotides in length that can be detected using probes specific for the sequences in color in **Figure 2**. However, those candidate siRNA sequences were neither cloned nor further characterized, and could, for example, represent cross-reactive cellular miRNA induced by HIV-1 infection. Given such considerations and the data reported above[37] indicating that HIV-1 does not in fact express any siRNA or miRNA in infected cells, the available experimental data provide no convincing evidence in support of the hypothesis that viral infection of mammalian cells induces siRNA production.

Based on the three criteria outlined above, the final issue then becomes whether any vertebrate virus encodes an SRS. In fact, several

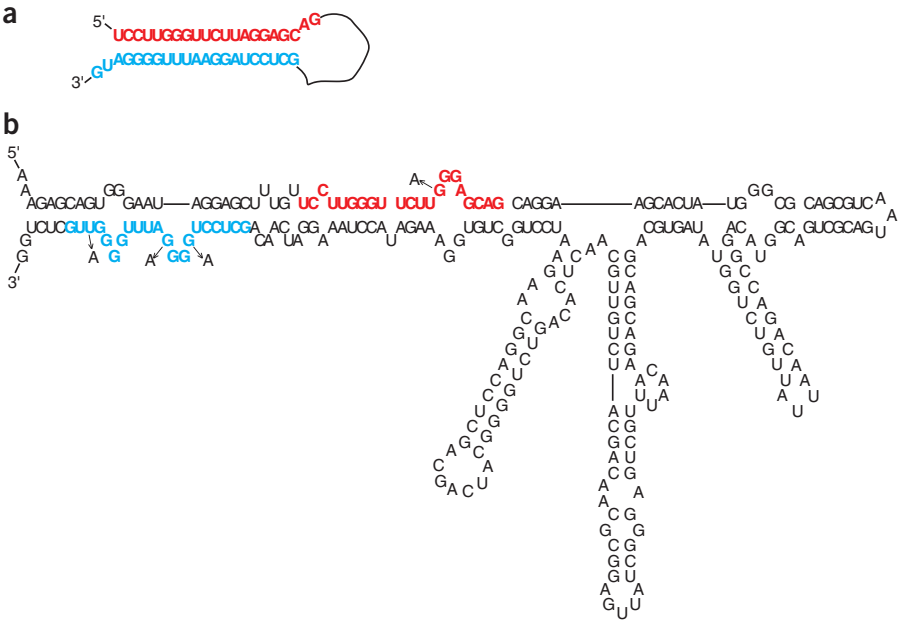

Figure 2 Proposed structures of a candidate HIV-1 siRNA precursor. (**a**) Bennasser *et al.*[38] propose that these two HIV-1 19-nucleotide sequences form a perfect RNA duplex. This represents an 'idealized' HIV-1 sequence that maximizes base pairing, including in particular substitution of A:U base pairs for what would otherwise be predicted to be less-stable G:U base pairs. The substrate used by Bennasser *et al.*[38] for *in vitro* Dicer processing is the RNA stem structure flanked by the two-nucleotide 3′ overhang and a short terminal loop. (**b**) Actual structure adopted by these same HIV-1 genome segments. The genome segments do not form base pairs in the RRE structure, which has been confirmed both *in vivo* and *in vitro*[40,41]. The segments are also separated by 197 nucleotides and are flanked on the 3′ and 5′ ends by several thousand nucleotides of mainly unstructured HIV-1 genomic RNA. Arrows indicate differences between this HIV-1 sequence, derived from the HXB-3 proviral clone, and the 'idealized' sequence modeled by Bennasser *et al.*[38] are indicated.

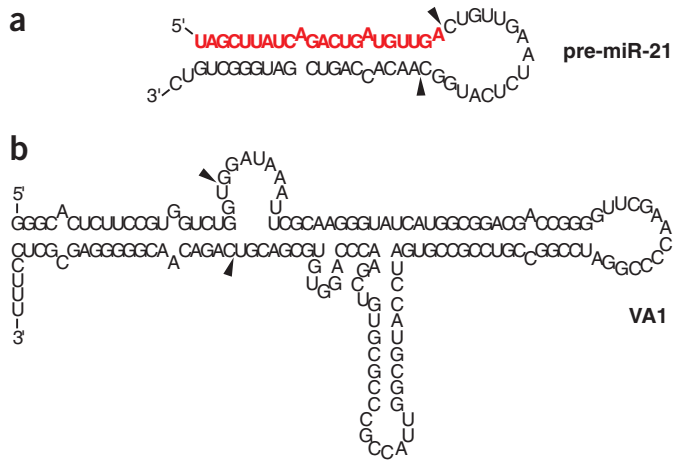

Figure 3 Comparison of the structures of a representative human pre-miRNA and adenovirus VA1 RNA. (**a**) Proposed structure of the human pre-miR-21 miRNA processing intermediate. The mature miR-21 sequence is in red. (**b**) Proposed structure of adenovirus VA1. Note that both have a terminal stem of 16 base pairs or more and a short 3′ overhang, which are required for binding by Exp5. Black arrowheads indicate known Dicer cleavage sites in pre-miR-21 and hypothetical cleavage sites in VA1.

candidates have been proposed and many these are supported by important data. One well established SRS is not in fact a virus-expressed protein but a virus-encoded RNA, the adenovirus VA1 RNA, approximately 160 nucleotides in length[45,46] (**Fig. 3b**). VA1 has extraordinarily high expression during adenovirus infection (up to 1×10^8 copies per cell) and functions as a potent inhibitor of the interferon-induced antiviral defense factor PKR. Data indicate that VA1 can also act as an effective competitive inhibitor of two key steps in the miRNA-siRNA biogenesis pathway: the Exp5-dependent nuclear export of pre-miRNA or shRNA and Dicer function[45,46] (**Fig. 1**). VA1 is bound and processed by Dicer, albeit very inefficiently, to give rise to RISC complexes containing VA1-derived miRNA[44,45].

Given those observations, the following question arises: Did VA1 evolve to be a true SRS or is its activity as an RNAi inhibitor coincidental? VA1 is a short RNA, produced in the nucleus by RNA polymerase III, whose nuclear export is mediated by Exp5, the factor used by pre-miRNA[7,8,47]. Recognition of an RNA export substrate by Exp5 requires a terminal helix of 16 base pairs or more with a short 3′ overhang, both of which are found in the VA1 structure[9,47] (**Fig. 3b**). Once VA1 reaches the cytoplasm, it then encounters the Dicer-TRBP complex, which binds short 3′ overhangs at the base of RNA stems of 19 base pairs or more; that is, a structure closely resembling the one required by Exp5 (refs. 12,19). The observed VA1-Dicer interaction, therefore, could reflect the overlapping RNA structural requirements for recognition by Exp5. Given the extremely high expression of VA1 that occurs during adenovirus infection, the inhibitory 'function' of VA1 therefore could be inadvertent. Thus, at present, the issue of whether or not the RNAi-inhibitory activity of VA1 specifically evolved remains unresolved.

A second candidate viral SRS is the NS1 protein encoded by influenza virus. NS1 is a potent inhibitor of the interferon system during influenza infection and has a well defined dsRNA-binding domain that is essential for activity[20]. Although overexpression of influenza NS1 can inhibit the induction of RNAi in both insect cells and plants[48–50], no evidence exists indicating that NS1 inhibits RNAi during influenza infection of mammalian cells. Although the results obtained in plants and insects might be viewed as strong evidence that NS1 is an SRS, it has been demonstrated that proteins that randomly bind dsRNA, such as bacterial RNase III, can also block RNAi in plants[51]. As the only domain in NS1 required for SRS function is in fact its 82–amino acid dsRNA-binding domain[48], it seems likely that a nonspecific mechanism underlies its observed SRS activity. Of note, whereas influenza virus mutants lacking NS1 are replication incompetent in normal cells and in wild-type mice, influenza NS1 mutants replicate effectively in mutant cells lacking a normal interferon response and are highly pathogenic in mice that lack the ability to mount an interferon response[52]. These data suggest, therefore, that the main and possibly only function of NS1 is to block the interferon response and that if NS1 has an SRS activity, it is dispensable for influenza pathogenesis.

A few other viral dsRNA-binding proteins, such as vaccinia virus E3L and reovirus σ3, have also been shown to inhibit RNAi in plants or insect cells[48,51], but the same issue of specificity as that described above remains to be addressed for these possible SRS proteins. Finally, two retroviral proteins, the Tas protein encoded by primate foamy virus and the Tat protein encoded by HIV-1, have also been proposed to function as inhibitors of RNAi[38,53]. As both Tas and Tat are nucleic acid–binding proteins that act as nuclear transcriptional activators, it is not immediately apparent how they could affect cytoplasmic RNAi. The data supporting Tas as an SRS again rely mainly on data from plant model systems in which, as noted above, overexpression of any dsRNA-binding protein seems to inhibit RNAi[51]. It has in fact been suggested that Tas may function mainly as an inhibitor of cellular miRNA molecules that, by evolution or fortuitously, show homology to regions of the primate foamy virus RNA genome[53]. Whether Tas indeed acts as an SRS in primate foamy virus–infected cells remains unclear.

The last candidate mammalian viral SRS is the HIV-1 Tat protein, which was proposed to have such a function in the report that also suggested that HIV-1 encodes a virally derived siRNA[38]. As noted above, it seems unlikely that such an HIV-1-derived siRNA is made, and even if it were produced, it would probably be unable to bind its proposed highly structured RNA target (**Fig. 2**). The proposed benefit to HIV-1 in expressing an SRS (protection from RNAi induced by this purported viral siRNA) is therefore unsubstantiated. Moreover, the main evidence in favor of that hypothesis involves massive overexpression of the HIV-1 Tat protein, which can act as a nonspecific dsRNA-binding protein[54]. Therefore, the reported SRS activity of HIV-1 Tat in mammalian cells may be analogous to the reports of nonspecific SRS activity of overexpressed dsRNA-binding proteins in plant cells[51]. Indeed, with more physiological amounts of either Tat or Tas expression, neither protein demonstrates detectable SRS activity (J. Lin and B.R. Cullen, unpublished results).

Conclusion

Although data supporting the conclusion that RNAi represents an important component of innate antiviral immunity are persuasive for plants and invertebrates, at present the evidence fails to support that hypothesis for vertebrates. No convincing data supporting the production of virus-derived siRNA in infected vertebrate cells and good evidence against its existence has been reported. Moreover, although several proteins or RNA molecules derived from human viruses can function as SRS proteins in heterologous systems or even when overexpressed in human cells, there is at present no evidence indicating that such SRS activity is physiologically relevant during virus infection. In fact, several viruses have now been shown either to express their own miRNAs in infected cells or to take advantage of host cell miRNAs to enhance their replication[37,55,56]. It therefore seems reasonable to propose that the extremely potent interferon system has displaced RNAi as the key defense against virus infection in mammalian cells[20] and that

RNAi now exists in vertebrates only as a mechanism of post-transcriptional regulation 'programmed' by endogenously encoded miRNA[3]. In certain 'artificial' conditions, however, such as after the introduction of exogenous nucleic acids with precise structural characteristics, the vertebrate RNAi machinery can be 're-programmed' to render cells resistant to virus replication[2]. Thus, inducing such 'artificial' RNAi responses may yet emerge as an important approach to the treatment of viral infections in mammalian cells.

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COMPETING INTERESTS STATEMENT

The author declares that he has no competing financial interests.

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