siRNA, miRNA and HIV: promises and challenges

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ABSTRACT

Small interfering RNA (siRNA) and microRNA (miRNA) are small RNAs of 18-25 nucleotides (nt) in length that play important roles in regulating gene expression. They are incorporated into an RNA-induced silencing complex (RISC) and serve as guides for silencing their corresponding target mRNAs based on complementary base-pairing. The promise of gene silencing has led many researchers to consider siRNA as an anti-viral tool. However, in long-term settings, many viruses appear to escape from this therapeutical strategy. An example of this may be seen in the case of human immunodeficiency virus type-1 (HIV-1) which is able to evade RNA silencing by either mutating the siRNA-targeted sequence or by encoding for a partial suppressor of RNAi (RNA interference). On the other hand, because miRNA targeting does not require absolute complementarity of base-pairing, mutational escape by viruses from miRNA-specified silencing may be more difficult to achieve. In this review, we discuss stratagems used by various viruses to avoid the cells' antiviral si/mi-RNA defenses and notions of how viruses might control and regulate host cell genes by encoding viral miRNAs (vmiRNAs).

Keywords: small interfering RNA, microRNA, RNA interference, human immunodeficiency virus type-1, RNA-induced silencing complex, suppressor of siRNA, viral miRNA.

INTRODUCTION

The recent discovery of small interfering RNA (siRNA) revealed an important role for small RNAs in regulating gene expression. First described in plants, as "post-trancriptional gene silencing" (PTGS) [1], RNA interference (RNAi) is a nucleic-acid based immune defense against viruses, transgenes and transposons [2]. Triggered by double-stranded RNA (dsRNA), RNAi leads to the sequence specific degradation of a target mRNA [3]. In eukaryotic cells, long dsRNAs are processed by Dicer, an RNAse III like protein, into small RNA duplexes of 21 to 23 nucleotides, called siRNA [4, 5] (Fig. 1). A cellular protein called TAR RNA-binding protein (TRBP) then interacts with and shuttles the Dicer-siRNA complex into the RNA-induced silencing complex (RISC) which contains another protein called Argonaute 2 (Ago2) [6, 7]. While other components of RISC remain to be identified, the current view is that one processed strand of the siRNA is incorporated into the protein complex and serves as a guide sequence for recognition of target RNAs. Thus, an active RISC consists minimally of the Ago2 protein and the single-stranded siRNA guide [8-11]. When a perfect base-pairing of the single-stranded guide RNA with the target mRNA is made, the PIWI domain of the Ago2 protein is brought into proximity and cleaves the target mRNA resulting in a post-transcriptional gene silencing [12, 13].

In addition to post-transcriptional RNA silencing, components of the RNA interference pathway are also postulated to be involved in transcriptional gene silencing through RNA-dependent DNA methylation [14]. siRNAs which originate from bidirectional transcription of a cell's target loci are incorporated into an RNA-induced initiator of transcriptional gene silencing (RITS) complex. The composition of RITS is variable from one species to another, and its components are not completely identified. In plants and fission yeast, RITS includes Dicer, Ago1 (an Argonaute

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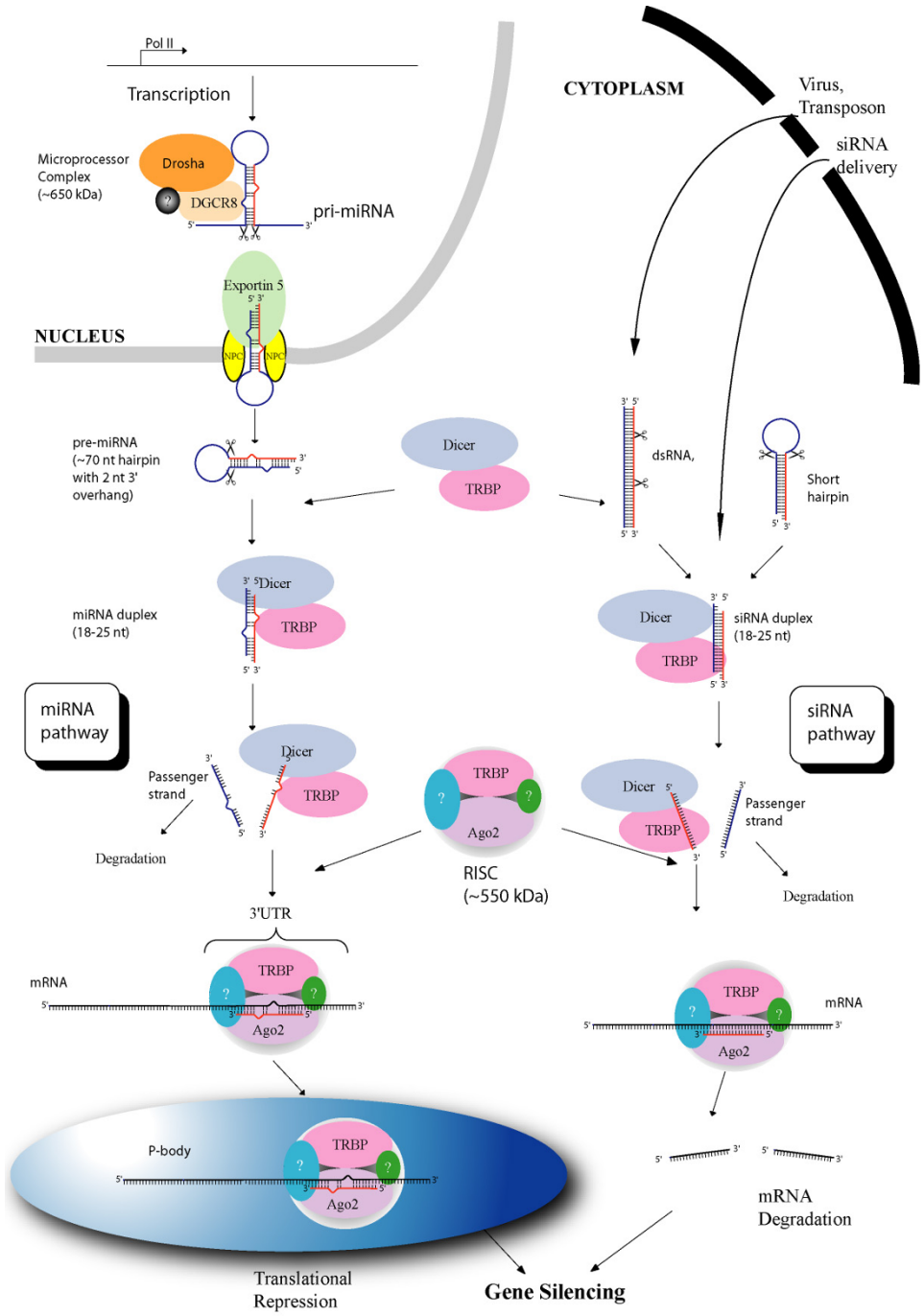

Fig. 1 A comparison of the miRNA and siRNA pathways. miRNAs are derived from large highly-structured precursors pri-miRNAs transcribed by RNA Pol II in the nucleus. After cropping by the Microprocessor, a complex containing Drosha and DGCR8, the pri-miRNAs are processed into ~70 nt hairpin with 2 nt at the 3' overhang (pre-miRNAs). With the aid of Exportin 5, the pre-miRNAs are then exported into the cytoplasm. In the cytoplasm, both pre-miRNAs and viral/transposon-derived dsRNAs/short hairpins share a common processing pathway which mainly involves Dicer/TRBP and RISC. The loop regions of pre-miRNA and the dsRNAs/short hairpins are trimmed by Dicer to generate miRNA and siRNA duplexes, respectively. One strand of the duplexes is incorporated into the RISC and serves as a guide for RNA silencing. The remaining RNA strand (passenger strand) is then targeted for degradation. Although both miRNAs and siRNAs utilize RISC for gene silencing, mRNAs with perfect complementary to the siRNAs are targeted to degradation while transcripts captured by miRNA-incorporated Ago2 are directed into the P-body resulting in translational repression. NPC: Nuclear Pore Complex

homolog), Chp1 a chromodomain protein and an RNA-dependent RNA polymerase (RdRP) which so far has no known equivalent analogue in vertebrates or insects [15]. It remains unclear whether RITS binds chromatin through a targeted nascent transcript, or through DNA, and then it is envisioned that a protein complex including a methyltransferase that methylates histone 3 methyl 9 (H3K9) is recruited to the promoter. H3K9 methylation consolidates RITS/chromatin complex and initiates the methylation of CpG islands resulting in transcriptional repression. Although apparently well-described in lower eukaryotes; currently, it remains uncertain whether RITS is fully operative in higher eukaryotes.

Similar to siRNA, another kind of small RNA, microRNA (miRNA) of 18-25 nt, has also been identified to interfere with gene expression [16-18]. miRNAs were first described in *Caenorhabditis elegans* (*C. elegans*) to regulate developmental timing [19]. To date, the miRNA Registry database (http://www.sanger.ac.uk/Software/Rfam/mirna) records 2909 miRNA sequences including 78 in *Drosophila melanogaster*, 114 in *C. elegans*, 321 in *Homo sapiens* [20]. A miRNA is derived from the transcription of a large highly-structured precursor (pri-miRNA) of ~ 70 nt encoded by a cellular gene. The pri-miRNA is then processed in the cell's nucleus into an imperfect shorter stem-loop structure (pre-miRNA) by the Microprocessor, a large complex that includes Drosha and the RNA binding protein DGCR8 [21]; pre-miRNAs are then exported into the cytoplasm by Exportin 5 (Fig. 1). In the cytoplasm, the loop region of the pre-miRNA is cleaved by Dicer to generate an imperfect dsRNA of 18-25 nt in length. Like siRNA, one strand of the double-stranded miRNA is incorporated into the RISC which then becomes armed to silence gene expression [22]. In contrast to siRNA, perfect complementarity is not necessary for miRNA to target its transcripts. One miRNA is, therefore, able to target a number of different transcripts. Thus, a microarray analysis has recently shown that the cellular miRNA, miR-124, can regulate up to ~100 mRNAs [23]. Conversely, various miRNAs can silence a gene by targeting different portions of the same mRNA. Although both siRNA and miRNA utilize the same machinery (RISC) for gene silencing, the fates of their target mRNAs are not the same. siRNAs shut down gene expression at a post-transcriptional level through mRNA degradation, while miRNAs silence their target genes mainly through translational repression. Once the target transcript is bound to the miRNA-armed Ago2 (within RISC), the entire complex is directed into a ribosome-free compartment named "P-body" [24, 25]. Sequestration of the transcript into the P-body results in a blockage of translation (Fig. 1).

RNA INTEFERENCE: HIV-1 AS AN EXAMPLE

Recently, RNA silencing has become widely adopted as an experimental tool to inhibit the expression of cellular and viral genes. Accordingly, several siRNAs have been designed to target HIV infection [26]. The first HIV targets were essential genes: such as the *gag* structural protein [27], the infectivity factors *vif* and *nef* [28], and the post-transcriptional regulatory protein, *rev* [29]. Using siRNA, many investigators reported sequence-specific silencing of targets and observed a robust inhibition of HIV replication during single round infection or short term tissue culturing of virus spanning 3 to 4 days. In addition to targeting HIV genes (*nef, tat, gag, vif, env*) [26, 30, 31], some have also targeted cellular proteins important to the HIV-1 life cycle. Thus, siRNAs were designed and tested against different steps of virus replication: i) at the entry level to HIV coreceptors CCR5 and CXCR4 [32-34]; ii) at the pre-integration step to the Arp2/3 complex responsible for actin polymerization involved in the routing of HIV toward the nucleus [35], and to PARP-1 [poly (ADP-ribose) polymerase-1] an abundant nuclear enzyme required for HIV integration [36]; iii) to cyclin T1, CDK9 and SPT5 which function at the level of HIV transcription for elongation of RNA polymerase II on the HIV-LTR [37, 38], and iv) to other key components such as cyclophilin A [39] and cdk2 [40]. All these targets were found to be efficiently inhibited by transfection of specific siRNAs, and their inhibition correlated with reduction in HIV replication in tissue culture [41]. However, one potentially noteworthy limitation to these findings is the relatively short duration of the assays for efficacy. Indeed, contrary to PTGS in plants which have an auto-amplification step, in mammals the effects of siRNAs are transient. One major obstacle remains how to continuously express siRNA in cells and whether sustained inhibition of viruses can be achieved in mammalian cells. A solution for continuous expression is addressed partially by the use of short hairpin sequences (shRNA) which can be processed intracellularly by the cell's Dicer [29, 42]. Whether sustained silencing can be durably maintained by exogenously introduced shRNA-expression vectors remain to be established.

ESCAPE OF HIV-1 FROM RNA INTERFERENCE

We and others have recently reported that HIV can escape from siRNA-mediated gene silencing. Many studies have described that HIV escapes from RNA interference through nucleotide mutations [43]. Das *et al*. observed that long term targeting of *nef* sequence using a lentiviral vector which expressed a suppressive shRNA eventually

selected for HIV with nucleotide mutations that escaped RNA interference [44]. Sequencing of the emerged RNAi-resistant virus showed that the *nef* sequence was altered in several ways: in some cases the siRNA recognition site contained several nucleotide substitutions which disrupted base-pairing and in other cases the target was simply deleted. Similar mutation patterns were also observed when Tat was targeted by exogenously introduced shRNA in a stable cell line [45]. Intriguingly, in a more recent report, a different mutational strategy to escape from RNAi was revealed by HIV-1. In this latter study, mutated viruses which showed high level of resistance to RNAi, evolved substitutions, near or in the targeted RNA sequence, which induced an alternative RNA folding resulting in a shielding of the target sequence from recognition by the guide RNA in RISC [46].

How to counteract the viruses' ability to mutate and escape from RNAi is a prime challenge which impedes the development of an efficient and long lasting anti-viral therapy. One idea is to administer several siRNAs targeted to different viral sequences simultaneously. In the case of hepatitis C virus (HCV) the combined use of two siRNAs limited the emergence of escape mutants seen when each siRNA was used separately [47]. Recently, a computational model was designed to predict viral mutations generated by HIV infection of cells which express RNAi targeted to the virus [48]. Such a tool may be helpful for designing multiple anti-HIV siRNAs which could be used combinatorially [49]. Indeed, the ability of HIV to escape from siRNA reflects another facet of the well-known mutability of this virus for changing its amino acid sequence to avoid immune clearance *in vivo* [2]. Unless better strategies are employed, all extant data suggest that singular targeting of individual siRNAs to HIV will always provoke rapid emergence of resistant virus [50-52].

Because HIV-1 appears able to escape facilely from

Tab. 1 Summary of the known virus-encoded suppressors in plant and animal viruses

	Virus	Virus family
Plant viral suppressors of RNA silencing		
Beta- C1	Tomato leaf curl china virus	Begomavirus
CP	Turnip crinkle virus	Carmovirus
S protein	Cowpea mosaic virus	Comovirus
p21	Beet yellows virus	Closterovirus
2b	Cucumber mosaic virus	Cucumovirus
virus P14	Beet necrotic yellow vein	Furovirus
AC2	African cassava mosaic virus	Geminivirus
C2	Mungbean yellow mosaic virus	Geminivirus
gb	Barley stripe mosaic virus	Hordeivirus
P15	Peanut clump virus	Pecluvirus
Pns10	Rice dwarf phytoreovirus	Phytoreovirus
PO	Beet western yellows virus	Polerovirus
p25	Potato virus X	Potexvirus
HC-Pro	Potato virus Y	Potyvirus
P1	Rice yellow mottle virus	Sobemovirus
NS3	Rice hoja blanca virus	Tenuivirus
P19	Tomato bushy stunt virus	Tombusvirus
p30	Tobacco mosaic virus	Tombamovirus
NSs	Tomato spotted wilt virus	Tospovirus
p69	Turnip yellow mosaic virus	Tympvirus
Animal viral suppressors of RNA silencing		
VA1	Adenovirus	Adenovirus
Tat	Human Immunodeficiency Virus	Retrovirus
Tas	Foamy virus type 1	Retrovirus
B2	Flock house virus	Nodavirus
NS1	Influenza A	Orthomyxovirus
E3L	vaccinia virus	Poxvirus

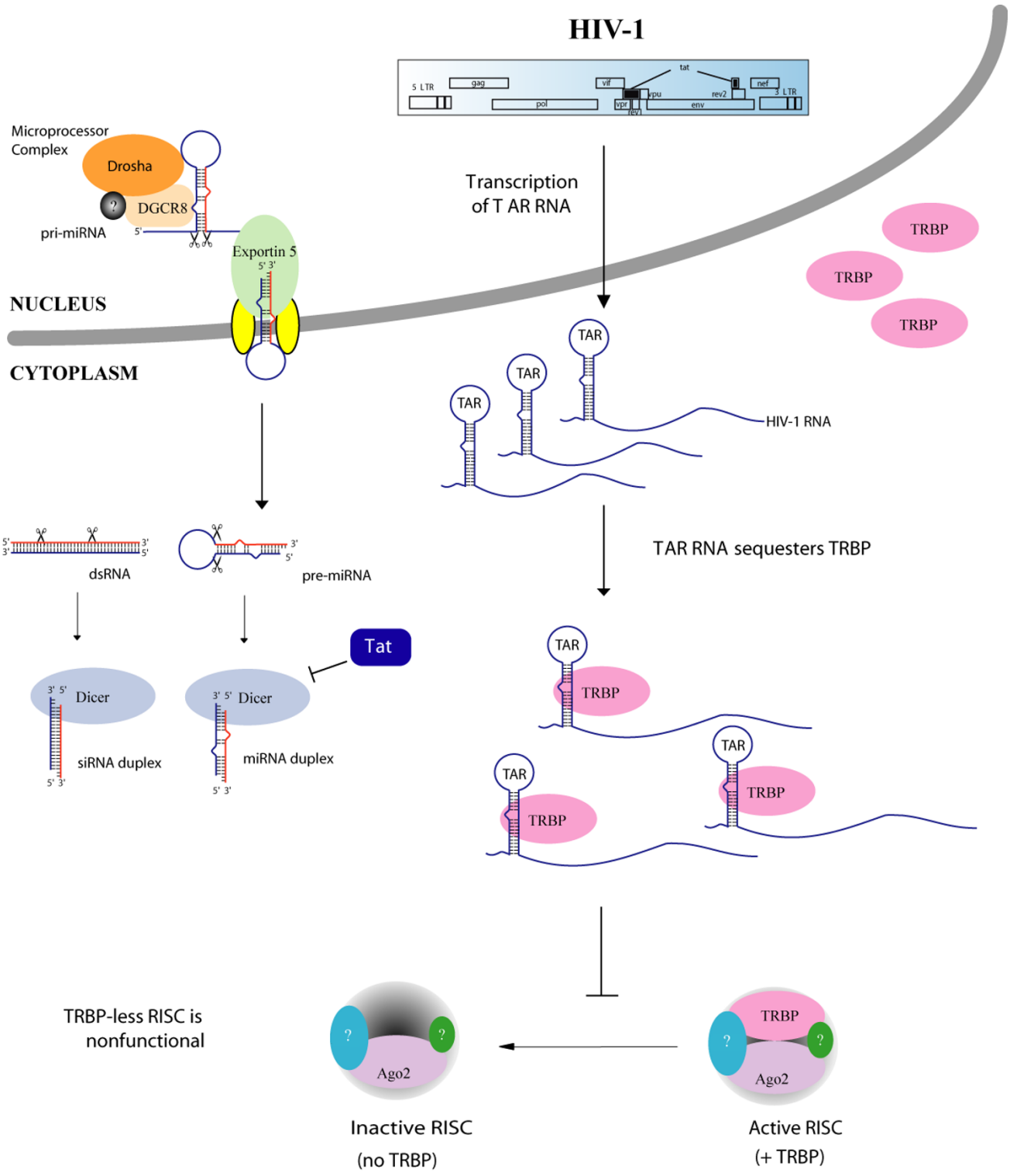

Fig. 2 Interference of the RNA silencing pathway by HIV-TAR RNA. TRBP is a Dicer-interacting protein which leads the Dicer-processed dsRNA to Ago2 in the RISC. In HIV-1 infected cells, TRBP is envisioned to be sequestered by TAR, a small hairpin leader RNA which is abundantly transcribed by HIV-1 (right). TAR-mediated depletion of TRBP prevents the formation of a functional RISC. On the left, a schematic is shown in which HIV-1 encoded Tat protein is postulated to directly interfere with Dicer function and thus leading to the suppression of the RNA silencing pathway.

exogenously targeted siRNA, it was highly unexpected that when we used a computer-directed systematic search over the entire HIV-1 genome, we found that the virus maintained a single region of duplexing that could serve as a virus-endogenous shRNA (vshRNA) [50]. Overexpression of this vshRNA was found to inhibit HIV replication. Because this region is conserved among different HIV strains, we hypothesize that there may be unknown selective pressure as to why HIV has not mutated this duplex. If there is indeed a functional constraint that prohibits HIV-1 from altering this vshRNA duplex, then this site could represent an "immutable" sequence to which exogenously introduced siRNA could be targeted efficaciously and durably.

If a virus contains a double-stranded RNA sequence that it cannot mutate, then in order for the virus to escape an induced RNAi, it likely needs to encode an RNAi-suppressor. In addition to its ability to generate nucleotide mutations, HIV also appears to utilize a suppressor-strategy to combat the cell's RNAi-defense. This latter discovery came to us serendipitously; our experimental findings suggested that the HIV-encoded Tat protein works to suppress RNA silencing in human cells [50]. Mechanistically, we observed that Tat inhibits Dicer activity *in vitro*. In the context of intracellular infection, Tat's inhibition of siRNA-processing by Dicer is partially effective as evidenced by the fact that a Tat-deleted virus processed the virus encoded vshRNA more effectively than a Tat-expressing wild type HIV-1 [50]. We note with great interest that a retrovirus related to HIV, human T-cell leukemia virus (HTLV-1) [53], has been described to encode an anti-sense transcript in the 3' end of its genome [54]. While the exact function of this transcript remains to be fully clarified, we would predict that this HTLV-1 anti-sense RNA would form with its counterpart sense viral transcript a double-stranded siRNA precursor. If our prediction is correct, then HTLV-1, like HIV-1, also maintains a viral siRNA. Moreover, by inference, HTLV-1 would need to encode (or co-opt) a yet-identified suppressor of RNAi.

The concept of a virus-encoded suppressor of RNAi is not new. The first RNA silencing suppressors were described in plant viruses {i.e. the P1/HcPro polypeptide in *Tobacco etch potyvirus* (TEV) [55] and p19 protein in *Tomato bushy stunt virus* (TBSV) [56]}. To date 20 viral suppressors of RNA silencing have been described in plant viruses and 6 in animal viruses [2, 57, 58] (Tab. 1). These proteins do not conserve any sequence homology with each other, and many are dsRNA binding protein. In addition to direct dsRNA binding which can be understood to inhibit the processing of siRNAs, the various suppressors appear to act at different steps of the RNA interference pathway. For example, after homodimerization, the p19 tombusvirus protein binds to siRNA and prevents its incorporation into the RISC [59, 60]. By contrast, the Potyviruses HcPro binds siRNA not to influence its association with RISC, but to block its functional activity [61].

One additional way that HIV might interfere with the RNA silencing pathway comes from the identification of TRBP as an essential component of the RNA interference machinery [6, 7, 62]. TRBP was first cloned and identified as a TAR RNA-binding protein [63]. TRBP interacts with Dicer and directs the siRNA–Dicer complex to Ago2 the catalytic component of RISC. Knockdown of TRBP results in destabilization of Dicer and a loss in miRNA biogenesis [6]. Because TAR RNA is a high-affinity ligand for TRBP, one can imagine that when HIV-1 expresses TAR RNA this viral RNA molecule could attach to TRBP and making it unavailable for use by the Dicer-siRNA-RISC complex. Thus both Tat and TAR may represent virus-encoded moieties that act to suppress Dicer and/or RISC function (Fig. 2).

FUNCTIONAL ROLES FOR KNOWN MIRNA

Although similar to siRNA, miRNAs are derived from endogenous RNAs that contain stem-loop structures. miRNAs have been described in a variety of species including invertebrates, plants, insects and mammals [64]. To date, 321 human miRNAs are listed in the miRNA Registry database. Some miRNAs function to regulate developmental timing, signal transduction, apoptosis, cell proliferation and tumorigenesis [65, 66] (Tab. 2). Interestingly, a cellular miRNA (miR-32) was recently identified to inhibit infection by the primate foamy virus type 1 (PFV-1) [67]. Knock-down of miR-32 expression by anti-sense oligonucleotides or deletion of the miRNA target sequence from the viral genome significantly enhanced PFV-1 replication. This finding provides the first evidence that cells may use miRNA as an anti-viral defense. Of interest, PFV-1 was found to encode a suppressor of miRNA in its Tas protein. Thus, it was found that Tas-expressing cells showed increased PFV-1 production when compared with the Tas-nonexpressing cells [67-69]. PFV-1's infectivity therefore depends on three factors: the tissue-specific miRNA expression pattern (i.e. miR-32), the expression of PFV-1 encoded viral silencing suppressor (i.e. Tas), and the ability of PFV-1 to mutate its viral sequence targeted by miR-32. Above, we pointed out the interesting parallel that HIV encodes a Tat protein which appears to partially suppress Dicer's function [50]. One intriguing unanswered question is whether there are some cellular miRNAs that target HIV, and if so whether HIV-1 Tat could also be a miRNA-suppressor.

In a different context, the human miRNA, miR-122 is

Tab. 2 Functions of known miRNAs

Biological Function of miRNAs	miRNA	Target gene(s)	Species	Refs
Developmental Timing	lin-4	lin-14	Ce	[87, 88]
		lin-28		[89]
	let-7	lin-41	Ce	[90, 91]
		hbl-1		[92, 93]
	lsy-6	cog-1	Ce	[94]
	miR-172	AP2	At	[68, 95-97]
	miR-JAW	TCP4	At	[98]
	miR-159	MYB33	At	[95, 98]
	miR-7	HLHm3	Dm	[99, 100]
		hairy		[100]
		m4		[100]
	miR-273	die-1	Ce	[101]
Apoptosis	miR-14	grim	Dm	[100, 102]
		reaper		[100, 102]
		sickle		[100, 102]
	bantam miRNA	hid	Dm	[103]
Cell proliferation	miR-101	ENX-1	Hs	[104]
		N-MYC		[104]
Tumorigenesis	miR-19a	PTEN		[104]
Signal transduction	miR-375	Myotrophin	Mm	[105]

Ce, Caenorhabditis elegans; At, Arabidopsis thaliana; Dm, Drosophila melanogaster; Hs, Homo sapiens; Mm, Mus musculus.

specifically expressed in liver cells and was found to be required for efficient Hepatitis C virus (HCV) replication in hepatocytes [70]. In this case, the miRNA is an activator of the viral infection. It is thought that miR-122 acts through binding to the 5' non-coding region of the HCV RNA, although miR-122's exact mechanism of action remains unknown. The effect of miR-122 appears to be tissue specific and not absolutely essential since in cells that do not express miR-122 (i.e. non-hepatic cells), HCV replication can be documented. It remains to be seen if miR-122 is a unique example or whether other cellular miRNAs could target 5' non-coding region of viruses and contribute to facilitated replication of pathogens.

IDENTIFICATION OF VIRAL MIRNA

Recent reports suggest that viruses can also encode miRNAs. To date, a total of 48 viral miRNAs (vmiRNAs) have been described (Tab. 3) in herpesviruses [71-73], polymoaviruses (SV40) [74] and retroviruses (HIV-1) [75, 76]. Most of the vmiRNAs were identified by small RNA cloning [71-73, 75]. A rare subset of vmiRNAs were predicted by computational algorithm and then verified by Northern blotting. In HIV-1, 5 stem-loop structures that can potentially be processed into miRNAs were predicted computationally in one report [77]. In a second report, a vmiRNA encoded by the *nef* region of HIV-1 termed miR-N367 was physically identified and isolated [75].

Size-fractionated small RNAs can be isolated and cloned from cells. Methodologically, RNAs of around 18-25 nt can be first purified by excision from acrylamide gel. To generate cDNAs from the purified small RNAs, individual linkers are ligated respectively to the 3' and 5' ends. Using the linkers, the small RNAs are reverse transcribed and then PCR amplified. Subsequently, restriction digestion of the linker sequences allows for the cloning of the small RNAs into plasmids. Sequencing of the cloned plasmids identifies small RNAs including miRNAs in cells.

A second way to identify miRNA is through computer-driven predications. Computational prediction uses an algorithm that recognizes the stem-loop structures present in the pri- and pre-miRNA. The program can scan large amounts of sequences and identify candidate stretches that can form stable local stem-loop structures. Based on the sequence and structural features conserved in known miRNAs, predicted miRNA-candidates are then selected and can be physically validated by Northern blotting. We

Tab. 3 Summary of the identified small RNAs encoded by viruses

Species	No. of Stem-loop	stem-loop	miRNA sequence
Epstein Barr virus (EBV)	5	ebv-miR-BART1	ucuuaguggaagugacgugcu
		ebv-miR-BHRF1-1	uaaccugaucagccccggaguu
		ebv-miR-BHRF1-2	uaucuuuugcggcagaaauugaa
		ebv-miR-BHRF1-2*	aaauucuguugcagcagauagc
		ebv-miR-BHRF1-3	uaacgggaaguguguaagcacac
		ebv-miR-BART2	uauuuucugcauucgcccuugc
Human cytomegalovirus	11	hcmv-miR-US5-1	ugacaagccugacgagagcgu
		hcmv-miR-US5-2	uuaugauaggugugacgauguc
		hcmv-miR-UL22A-1	uaacuagccuucccgugaga
		hcmv-miR-UL22A-1*	ucaccagaaugcuaguuuguag
		hcmv-miR-US25-1	aaccgcucaguggcucggacc
		hcmv-miR-US25-2-5p	auccacuuggagagcucccgcgg
		hcmv-miR-US25-2-3p	agcggucuguucagguggauga
		hcmv-miR-US33-1	gauugugcccggaccgugggcg
		hcmv-miR-UL36-1	ucguugaagacaccuggaaaga
		hcmv-miR-UL112-1	aagugacggugagauccaggcu
		hcmv-miR-UL148D-1	ucguccucccuucuucaccg
		hcmv-miR-UL70-1	ugcgucucggccucguccaga
		hcmv-miR-US4-1	cgacauggacgugcaggggga
Kaposi sarcoma-associated herpesvirus	12	kshv-miR-K12-2	aacuguaguccgggucgaucug
		kshv-miR-K12-1	auuacaggaaacuggguguaagc
		kshv-miR-K12-10a	uaguguugucccccgaguggc
		kshv-miR-K12-10b	ugguguugucccccgaguggc
		kshv-miR-K12-11	uuaaugcuuagccuguguccga
		kshv-miR-K12-3	ucacauucugaggacggcagcg
		kshv-miR-K12-3*	ucgcggucacagaaugugaca
		kshv-miR-K12-4-5p	agcuaaaccgcaguacucuagg
		kshv-miR-K12-4-3p	uagaauacugaggccuagcuga
		kshv-miR-K12-5	uaggaugccuggaacuugccgg
		kshv-miR-K12-6-5p	ccagcagcaccuaauccaucgg
		kshv-miR-K12-6-3p	ugaugguuuucgggcuguugag
		kshv-miR-K12-7	ugaucccauguugcuggcgcu
		kshv-miR-K12-8	uaggcgcgacugagagagcacg
		kshv-miR-K12-9	cuggguauacgcagcugcguaa
		kshv-miR-K12-9*	acccagcugcguaaaccccgcu
Mouse gammaherpesvirus 68	9	mghv-miR-M1-1	uagaaauggccguacuuccuuu
		mghv-miR-M1-2	cagacccccucucccccucuuu
		mghv-miR-M1-3	gaggugagcaggaguugcgcuu
		mghv-miR-M1-4	ucgaggagcacguguuauucua
		mghv-miR-M1-5	agaguugagaucgggucgucuc
		mghv-miR-M1-6	ugaaacugugugaggugguuuu
		mghv-miR-M1-7-5p	aaagguggaggugcgguaaccu
		mghv-miR-M1-7-3p	gauaucgcgcccaccuuuauu
		mghv-miR-M1-8	agcacucacuggggguuugguc
		mghv-miR-M1-9	ucacauuugccuggaccuuuuu
Human immunodeficiency virus Type 1	1	miR-N367	acugaccuuuggauggugcuucaa
Simian Virus 40	2	SVmiRNA	ugaggggccugaaaugagccuu
			gccuguuucaugcccugagu

used a computational approach slightly different from the standard miRanda software (http://www.microrna.org/miranda_new.html) [78] to predict the existence of miRNA candidates in the HIV-1 genome [77]. Once a vmiRNA is predicted, a second level of analysis can be made by scanning a database of human 3' untranslated mRNA regions for sites with complementarity to the vmiRNA. Such an analysis when applied to the 5 predicted vmiRNAs encoded by HIV-1 suggested the existence of ~500 to 1000 cellular transcripts that can be targeted by the virus [77]. If this analysis can be experimentally validated, then use of virally encoded miRNAs by viruses could be one way that these pathogens alter the landscape of host cell gene expression for their selfish benefit [79].

Despite the above hypothesis, the precise functions of many vmiRNAs are currently unknown. However, predictions of vmiRNA functions based on their potential target genes and their region of transcription have been made. One view is that vmiRNAs are used by viruses to self-moderate viral expression in infected cells; this moderation could assist such cells to escape immune surveillance [71, 74-76]. This idea is based on the finding that some of the vmiRNA sequences, including HIV-1 miR-N367 and EBV miR-BART2, target the coding regions of viral genes [71, 75]. By targeting their own transcripts, the viruses may attenuate their own pathogenicity and allow for maintenance of chronicity in host organisms. Indeed, ectopic expression of the miR-N367, a HIV-1 *nef*-derived miRNA, effectively silenced *nef* expression, and attenuated HIV-1's transcription and replication [75]. Similarly, miR-BART2, a miRNA derived from Epstein-Barr Virus (EBV), targets EBV's DNA polymerase (BALF5) for silencing [71]. Additionally, SV40-encoded miRNAs (SVmiRNAs) are expressed late in infection, and these SVmiRNAs have been found to regulate early viral mRNAs [74]. Interestingly, mutant SV40 deficient in SVmiRNAs accumulated excessive amounts of T antigen and became more susceptible to cytotoxic T lymphocyte (CTL) response as well as stimulated local cytokine release. Finally, more work is needed to understand how vmiRNAs can regulate cellular gene expression.

DIFFICULTIES IN ISOLATING HIV VIRAL MIRNA

Currently, the two common methods to identify miRNA are small RNA cloning and computational prediction followed by Northern blot analysis. The ability of these methods to detect miRNAs depends on the abundance of miRNAs and the accuracy of the software for predicting stem-loop secondary structures. Both criteria are challenging for vmiRNA identification because the timing and pattern of vmiRNA expression are unpredictable [79, 80]. Indeed, reports have suggested that some vmiRNAs are only seen during lytic viral replication (EBV, KSHV) [71, 82]. Thus, the ability to capture vmiRNA by small RNA cloning depends on the specific stage of infection, the robustness of infection, and the infected cell type.

Although good success in predicting eukaryotes miRNAs using computational programs has been reported [71, 72, 83, 84], sequence analysis of extant vmiRNAs show that each vmiRNA sequence is often unique and share no sequence homology with other vmiRNAs or with host cell miRNAs. Absent a collection of reliable rules, the accurate computational prediction of vmiRNAs is difficult because the current softwares for predicting cellular miRNAs do rely heavily on cross-species sequence conservation between cells [82, 84, 85]. An illustration of the difficulty with *in silico* prediction of viral miNRA is shown by the SVM methodology designed by *Pfeffer et al*. which predicted several herpesvirus vmiRNA [82], but was unable to provide useful vmiRNA predictions for small RNA viruses, including HIV-1. By contrast, 5 HIV-1 vmiRNA candidates were predicted by a different computational algorithm called *StemED* [77]. In addition to the predicted HIV-1 vmiRNAs, the physical isolation of an HIV-1 vmiRNA, miR-N367, by *Omoto et al*. [75] suggests that further understanding of the processes for vmiRNA biogenesis and the development of more sensitive and accurate tools for vmiRNA detection, prediction, and isolation are needed.

Finally, much attention has been focused on Drosha because of its role in miRNA maturation. Interestingly, Drosha also plays an important role in pre-ribosomal RNA processing [86]. We have made a curious observation that when we isolate small RNAs from cells, many isolates are fragments of rRNAs (unpublished observation). We note that others have also seen a high proportion of rRNA being isolated during their small RNA cloning attempts [71]. Whether Drosha authentically processes ribosomal RNAs into physiologically relevant small RNAs remains to be established. If the above thinking is found to be correct, then a provocative concept would be that rRNAs may also be precursors for functional small miRNA-like moieties. Perhaps, in addition to siRNAs and miRNAs, there is a third class of biologically active srRNAs, small ribosomal RNAs. Time will tell.

ACKNOWLEDGEMENTS

This work was supported through intramural funds from the NIAID, and the NCI, NIH.

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