

ANALYSIS OF THE INTERACTION BETWEEN VIRUSES, MIRNAS AND  
THE RNAI PATHWAY

by

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Date: March 26, 2008

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Dissertation submitted in partial fulfillment of  
the requirements for the degree of Doctor  
of Philosophy in the Department of  
Molecular Genetics and Microbiology in the Graduate School  
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ABSTRACT

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## **Abstract**

The microRNA (miRNA) and RNA interference (RNAi) pathways have recently emerged as an important aspect of virus-host cell interaction. This interaction can occur in several different ways and may favor either the virus or the host cell. Plants and invertebrates use RNAi as a first line of defense against virus infection by cleaving long, double-stranded viral transcripts into small interfering RNAs. However, it remains to be determined whether mammalian cells also initiate a similar response to infection. Here we present evidence that mammalian cells in fact do not induce an antiviral RNAi defense in response to infection by primate retroviruses.

Viruses may also interact with host cells by encoding miRNAs to regulate either cellular or viral gene expression. Here we demonstrate that herpes simplex virus type 1 (HSV-1) encodes at least five miRNAs which are primarily expressed during latency. Two of these miRNAs modulate expression of viral genes required for productive replication. We hypothesize that down-regulation of these viral genes by these latency-associated miRNAs allows HSV-1 to establish and maintain the latent state.

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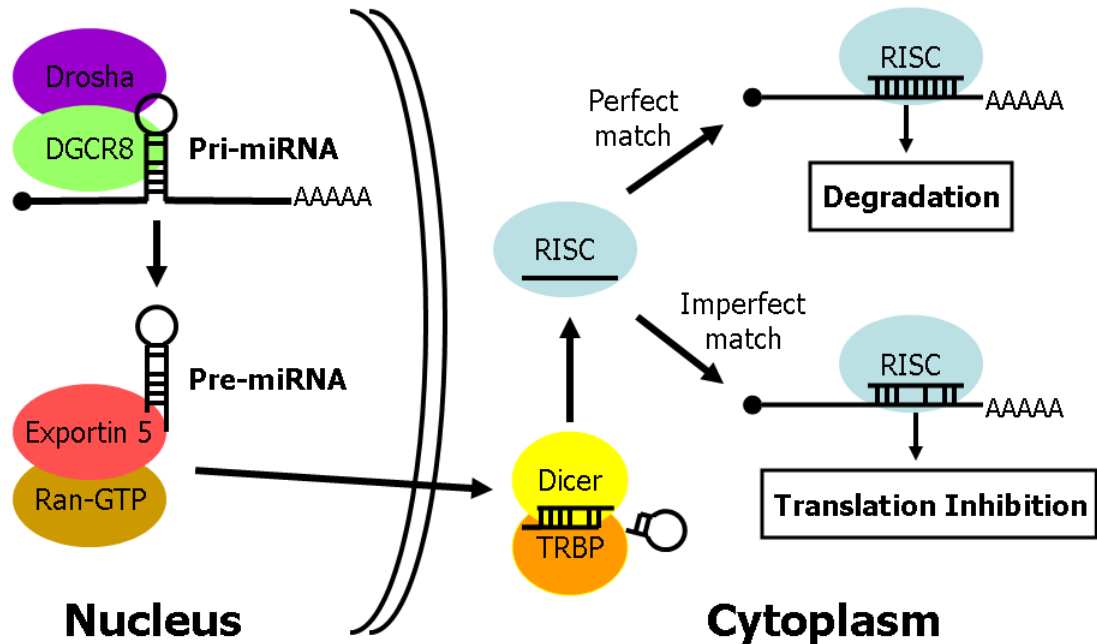
# 1. Introduction

## 1.1 *MicroRNA biogenesis*

The microRNA (miRNA) pathway is highly conserved [1], and plays a role in a wide variety of cellular processes including cellular proliferation [2], developmental regulation [3-6], apoptosis [7, 8], metabolic regulation [9] and tumor formation [10, 11]. Humans alone are known to encode over 500 different miRNAs [12, 13].

Cellular miRNAs are typically derived from a single arm of imperfect RNA hairpin structures encoded within capped and polyadenylated mRNAs referred to as primary-miRNA (pri-miRNA) transcripts [14, 15] (Fig. 1-1). These pri-miRNAs may be almost any size, from ~200 nts to thousands of kilobases and encode one or more miRNAs [16, 17]. In mammalian cells, these hairpin structures are excised from the pri-miRNAs by RNase III enzyme Drosha and its co-factor DGCR8 through cleavages 10-12 nt from the base of the stem to release an ~70 nt precursor-miRNA (pre-miRNA) [14, 18, 19]. Since the remaining 5' and 3' arms of the pri-miRNA are typically degraded after Drosha cleavage [15], pre-miRNAs are typically found within non-coding RNAs or the intronic regions of protein coding mRNAs [20].

Drosha cleavage results in a 2 nt overhang at the 3' end of the pre-miRNA [19, 21] which is recognized and bound by Exportin 5 and its co-factor Ran-GTP [22, 23]. The



**Figure 1-1. miRNA biogenesis pathway.** Capped and polyadenylated pri-miRNA transcripts are cleaved by Drosha-DGCR8 to release the pre-miRNA hairpin. Binding by Exportin 5-Ran-GTP exports the precursor to the cytoplasm where Dicer-TRBP cleavage removes the terminal loop, generating the miRNA duplex intermediate. The strand with lower binding energy at the 5' end is then loaded into RISC where it is used as a guide to target complementary mRNAs for inhibition. If the miRNA is perfectly complementary to a transcript, the target is cleaved and degraded; if the miRNA binds imperfectly, translation is inhibited.

heterodimer exports the hairpin from the nucleus to the cytoplasm where it is released upon hydrolysis of Ran. There, the same 2 nt overhang is recognized by Dicer [24], another RNase III enzyme, and its co-factor TRBP [19, 25]. Binding of Dicer-TRBP to the base of the pre-miRNA is followed by a downstream cleavage event that releases the terminal loop, resulting in a short, double-stranded RNA duplex ~21 nts long [24]. This duplex is passed onto the RNA induced silencing complex (RISC) [26-29] where the RNA strand with lower binding energy at the 5' end is loaded into the complex [30, 31]. The arm that is loaded is then used as a guide strand to target specific mRNAs for translational inhibition while the passenger strand is released and degraded [17].

Loaded RISC complexes typically bind to the 3'UTRs of target mRNAs in a cooperative manner; the greater number of complexes bound, the greater the inhibitory effect on translation [18, 29, 32, 33]. Typically, targeted mRNAs must match, at a minimum, nucleotides 2-8 of the guide strand, also referred to as the seed region [34, 35]. There are rare examples, however, where extensive base pairing between the target mRNA and the 3' half of the miRNA may compensate for mismatches in the seed region [36].

mRNAs perfectly complementary to the entire guide strand, however, are cleaved and degraded in a separate process referred to as RNA interference (RNAi) [27, 37, 38]. In this case, the small RNA guide is actually called a small interfering RNA (siRNA) [18, 39]. In contrast to miRNAs, siRNA binding may occur anywhere within the

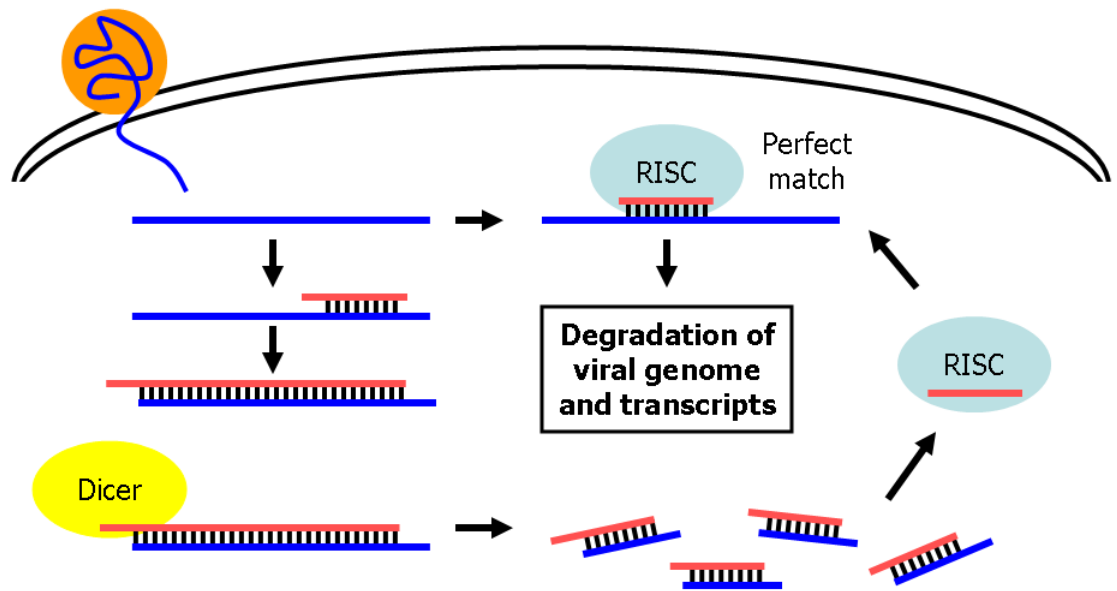
target transcript, including the coding region [40]. Moreover, as siRNAs act catalytically rather than cooperatively, a single binding site is sufficient to induce cleavage and degradation [29, 41].

Although there are no reported examples of antiviral RNAi occurring naturally in mammalian cells, RNAi may be potently induced through the artificial introduction of any intermediate of the miRNA processing pathway; plasmid-derived transcripts driven by RNA polymerase II (pol II) promoters may act as pri-miRNAs [15, 42], short hairpin RNAs (shRNAs) driven by RNA polymerase (pol III) promoters may be designed to mimic pre-miRNA hairpins [43], and short RNAs from synthetic duplexes are capable of being directly loaded by RISC [44].

## **1.2 Antiviral microRNAs and RNA interference**

In addition to using miRNAs for regulating cellular gene expression, cells also use miRNAs to defend against viral infection. For example, Otsuka *et al.* demonstrated that Dicer-deficient mice are hypersusceptible to infection by vesicular stomatitis virus (VSV) due a lack of cellular miRNAs miR-24 and miR-93 which are able to down-regulate expression of the viral large protein and phosphoprotein, respectively [45].

Cells may also use RNAi as a system of antiviral defense (Fig. 1-2). Infection by almost all RNA viruses leads to the generation of double-stranded RNAs (dsRNAs) at



**Figure 1-2. Antiviral RNAi pathway.** Almost all RNA viruses generate long, perfectly complementary dsRNAs as an intermediate step of the virus life cycle. In plants and invertebrates these RNAs are recognized as foreign by Dicer which cleaves them into ~21 nt siRNA duplexes. Individual arms of these duplexes are then loaded into RISC where they are used as templates to target viral genomes and transcripts for degradation.

some point during the virus life cycle. Unlike cellular dsRNAs which are relatively short and contain regions of mismatch, virus-generated dsRNAs may be extremely long, and are perfectly complementary. In plants and invertebrates, Dicer recognizes these foreign dsRNAs and processively cleaves them into ~21 nt siRNA duplexes. Individual arms of these duplexes are then loaded into RISC and used as guide strands to cleave complementary viral genomes or transcripts, thereby inhibiting viral replication [46, 47]. This antiviral siRNA response can also be amplified through the action of RNA-dependent RNA polymerases (RdRps) which can dramatically increase the pool of siRNAs available to RISC, potentially inhibiting viral spread [48]. The generation of viral siRNAs has been observed during infection of various invertebrate systems including VSV infection of nematodes [49, 50] and Flock House virus (FHV) infection of either *Drosophila* [47] or nematodes [51].

In addition to the innate immune response, mammals have also evolved a highly sophisticated system of adaptive immunity [52, 53]. Whether or not mammalian cells still make use of the relatively primitive antiviral RNAi mechanism remains to be determined.

### ***1.3 Virus-encoded miRNAs***

miRNAs have several features that make them particularly appealing for use by viruses. Firstly, and most obviously, they may be used to target specific cellular, or viral,

genes for down-regulation in order to establish a favorable replication environment for the virus [36, 54-56]. Secondly, the evolution of a miRNA complementary to a new target gene can be accomplished much more rapidly and easily than the evolution of a novel regulatory protein. Thirdly, miRNAs are small; the minimal space requirement for a pre-miRNA hairpin is < 200 nts [20] which is highly amenable to the tight space constraints placed on many viral genomes. Lastly, miRNAs are not antigenic; unlike proteins, which may elicit an immune response, miRNAs do not.

Just as viruses have co-opted many other cellular functions for their own benefit, a growing body of research in recent years has demonstrated that many viruses do in fact encode miRNAs. Thus far the *herpesviridae* family has been found to have the most miRNA-encoding members [57-61]. Other miRNA-encoding viruses include SV40 [54] and adenovirus [62] which, like herpesviruses, are both nuclear, DNA viruses. This reflects a consequence of the fact that the Drosha and DGCR8 proteins required for miRNA processing localize to the nucleus [19, 20]; viruses that replicate exclusively in the cytoplasm would not have access to this machinery and therefore could not express miRNAs even if they encoded them [63]. In addition, because miRNA processing results in the destruction of the entire pri-miRNA transcript except for the mature miRNA [15], it is unlikely that RNA viruses would encode miRNAs since processing would result in the destruction of the viral genome (or genomic minus strand).

Another consideration is the fact that miRNAs act on the mRNA level [1]. Even with complete translational inhibition, the existing pool of proteins must be significantly degraded before a phenotype may be observed. Depending on the stability of the protein in question, this process could take a significant amount of time, thus limiting the potential benefits of miRNAs to viruses with long life-cycles.

Although it is certainly possible that exceptions exist to any of these considerations, it does suggest that the type of viruses most likely to encode miRNAs would be nuclear, DNA viruses capable of persistent or latent infection.

## **2. Interaction of primate retroviruses with the human RNA interference machinery**

### ***2.1 Summary***

RNAi is a well-characterized antiviral defense pathway for plants and invertebrates. Whether this process also serves as an antiviral mechanism for mammals, however, remains to be determined. Two separate reports recently suggested that infection of mammalian cells with the human immunodeficiency virus type 1 (HIV-1) results in either the production of viral siRNAs or a viral miRNA, both capable of limiting virus replication. A parallel report also suggested that primate foamy virus (PFV) infection was limited by the presence of cellular miRNA miR-32. It was proposed that HIV-1 and PFV both counter these antiviral effects by utilizing their transcriptional transactivating proteins as suppressors of RNA silencing (SRS). Our examination of these issues, however, has determined that primate retroviruses are unlikely to generate either siRNAs or miRNAs during infection of mammalian cells. In good agreement with this finding, we also determined that none of the retroviral transactivating proteins examined, including HIV-1 Tat, PFV Tas, and the human T-cell leukemia virus type 1 (HTLV-1) Tax protein, have SRS properties.

## **2.2 Introduction**

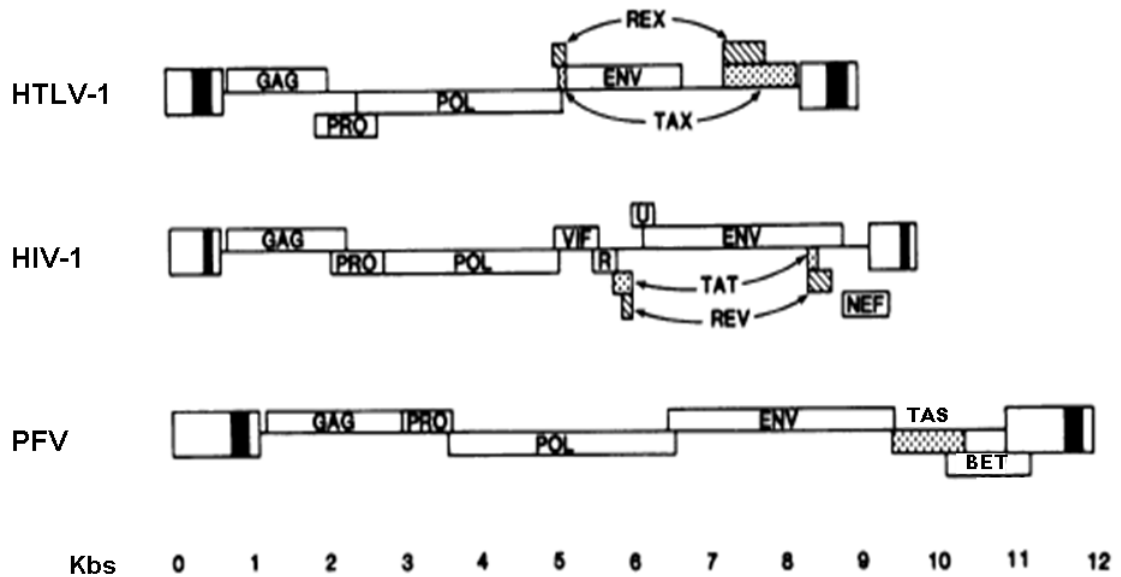
### **2.2.1 Retroviruses**

Retroviruses are small viruses with positive-strand RNA genomes (reviewed in [64]). Each virus contains two identical copies of the genome that are both capped and polyadenylated. Retroviruses may be broadly divided into two categories—“simple” or “complex”. Simple retroviruses encode only the Gag, Pro, Pol and Env gene products, whereas complex retroviruses also encode several additional small regulatory proteins.

HIV-1, HTLV-1 and PFV are a diverse group of complex primate retroviruses, each belonging to a different retroviral subfamily – deltaretroviruses, lentiviruses, and spumaviruses, respectively [64]. As a consequence, they each encode a different set of small regulatory proteins in addition to the standard retroviral gene products (reviewed in [65]) (Fig. 2-1).

Small regulatory proteins encoded by HIV-1 include Vif, Vpr, Vpu, Tat, Rev and Nef [66]. These genes control a variety of functions including viral transcription, transcript export, virion assembly and the inhibition of host antiviral factors. In contrast, HTLV-1 encodes only two small regulatory proteins—Rex and Tax, which are responsible for synthesis and nuclear export of viral transcripts [67]. Like HTLV-1, PFV also only encodes two regulatory proteins—Tas and Bet [68]

The hallmark feature of all retroviruses is a reverse-transcription step during the viral life cycle that converts the RNA genome into double-stranded DNA for integration



**Figure 2-1. HTLV-1, HIV-1 and PFV genome organization.** All complex retroviruses encode the Gag, Pro, Pol and Env proteins as well as various accessory proteins. Unmarked boxes flanking genomic sequences represent LTR promoters. (Figure adapted from [69].)

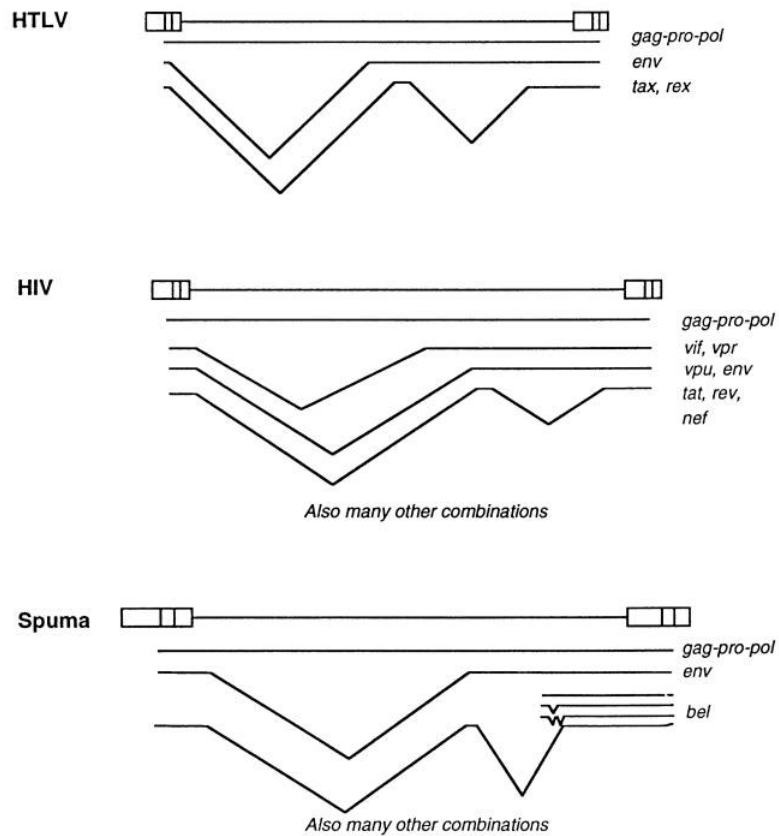
into the host genome [64]. The integrated genome then serves as the template for all subsequent viral transcription. This integration event is irreversible, and afterwards the viral genome is transmitted to all daughter cells as if it were a “normal” cellular gene. In this way, retroviruses are able to maintain persistent infections, permanently enter the germline, and induce the oncogenic activities of the retro-leukemia viruses (e.g., HTLV-1).

Reverse-transcription of the RNA genome into DNA results in the assembly of two long terminal repeat (LTR) promoters that flank both ends of the viral genome

(reviewed in [70]). The LTRs contain sequences recognized by RNA pol II which the virus utilizes to produce capped and polyadenylated viral transcripts [64]. Complex retroviruses produce three different species of transcripts—full-length, singly-spliced and doubly-spliced (Fig. 2-2). Full-length products encode the Gag, Pro and Pol proteins. Singly-spliced transcripts encode the Env protein, while the remaining small regulatory proteins are encoded by either singly- or doubly-spliced variants.

The two most important small regulatory proteins of HIV-1 are Tat and Rev. Tat is a potent transcriptional activator of the HIV-1 LTR [71]; it acts by binding to a hairpin structure located at the 5' end of the viral transcript known as the trans-activating responsive element (TAR) [72] (Fig. 2-3A). While bound to TAR, Tat recruits cellular co-factors cyclin T1 and Cdk9 to the RNA which act together to phosphorylate the polymerase C-terminal domain [73, 74]. These modifications are thought to enhance the processivity of the polymerase, thereby enabling it to transcribe the entire viral genome (Fig. 2-3B and C).

HIV-1 Rev is responsible for exporting unspliced and singly-spliced viral RNAs from the nucleus to the cytoplasm for translation [75]. Rev acts with its cellular co-factor Crm1 by binding to a large secondary structure referred to as the Rev response element (RRE) located within the *Env* gene of the viral transcript and exporting the RNAs out of the nucleus [76, 77]. In this way, the virus is able to generate the late structural Gag, Pol, Pro and Env proteins from genes which would otherwise be spliced out of the viral



**Figure 2-2. Complex retroviral transcript splicing patterns.** All complex retroviruses produce three species of transcript—full-length, singly-spliced and doubly-spliced. Late structural genes Gag, Pro and Pol are encoded on the full-length transcript while Env is located on the singly-spliced variant. Other accessory proteins are located on either the singly- or doubly-spliced transcript. (Figure adapted from [64].)

transcripts. Tat and Rev (along with Nef) are both encoded by doubly-spliced transcripts which do not contain an RRE and are therefore expressed as early genes.

The Tax protein of HTLV-1, similar to HIV-1 Tat, is also a transcriptional activator of its cognate viral LTR [78, 79]. Unlike Tat however, which is an RNA-binding protein that works by enhancing the elongation properties of polymerase, Tax is a

DNA-binding protein that works by increasing the rate of transcription initiation.

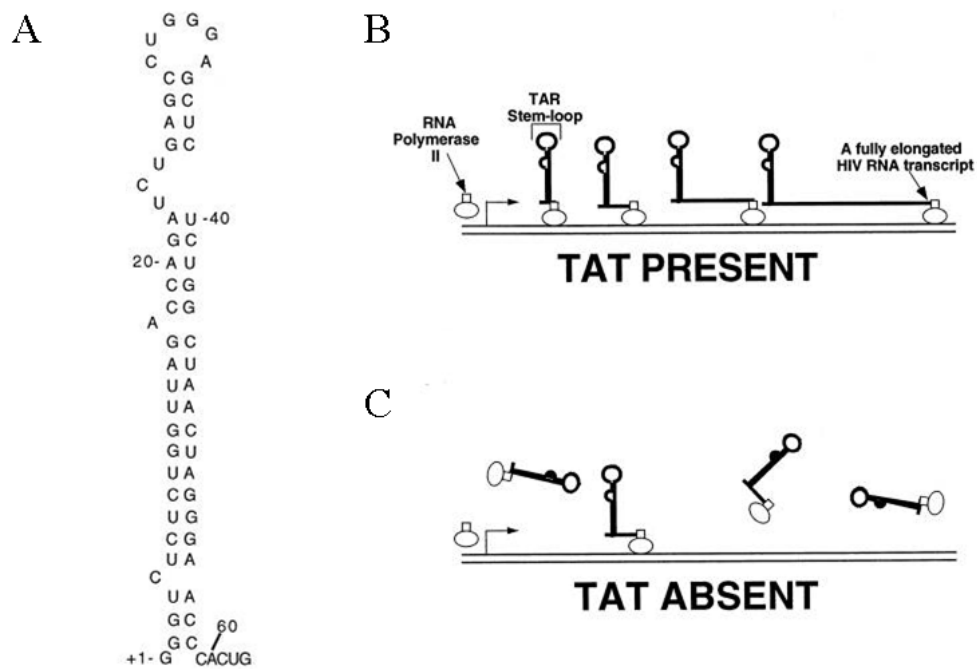
Meanwhile, the function and mechanism of the HTLV-1 Rex protein is similar to that of the HIV-1 Rev protein [80]. (Rex can, in fact, functionally replace Rev in HIV-1 although the reciprocal is not true).

Although PFV Tas is functionally equivalent to both HIV-1 Tat and HTLV-1 Tax [69], it operates via a different mechanism. Like Tax it is a DNA- rather than RNA-binding protein [81], but in addition to the PFV LTR, Tas also binds to an internal promoter (IP) site located within the 3' end of the *Env* gene [82, 83]. Tas binds the IP with a higher affinity than the LTR, therefore transcripts driven by the IP appear earlier during infection than transcripts driven by the LTR [82]. Since the IP drives transcription of the *tas* mRNA, Tas is able to stimulate its own production through a positive feedback loop. Eventually, enough Tas is accumulated to activate the LTR promoter and initiate transcription of the late structural genes. (The PFV Bet protein is functionally equivalent to the HIV-1 Vif protein.)

### **2.2.2 Antiviral miRNAs or siRNAs**

The question of whether or not retroviruses interact with the cellular miRNA/RNAi machinery of mammalian cells has been controversial [84]. Pfeffer *et al.* were the first to examine this issue by analyzing 18-24 nt cDNAs cloned from cells infected with HIV-1, yellow fever virus (YFV) or hepatitis C virus (HCV) [57]. Despite

sequencing at least 1,000 cDNAs from each library, no viral miRNAs or siRNAs were recovered from any of the samples.



**Figure 2-3. Structure and function of TAR.** A) Secondary structure of the TAR hairpin located at the 5' end of all HIV-1 transcripts. Tat binding to TAR recruits cellular co-factors cyclin T1 and Cdk9 to phosphorylate the polymerase CTD, thus enhancing the elongation properties of the polymerase. B) When Tat is present, RNA pol II is able to elongate and generate full-length viral transcripts. C) When Tat is absent, the polymerase is unable to elongate efficiently, and short, abortive transcripts are generated. (Figure adapted from [66].)

Recently, however, it was suggested by Bennasser *et al.* that a human T-cell line infected with HIV-1 generates a virus-specific siRNA capable of inhibiting productive infection [85]. A separate report by Omoto *et al.* suggested that HIV-1 actually encodes a miRNA derived from the *nef* gene that down-regulates Nef production thereby keeping HIV-1 viremia levels low in long-term non-progressing (LTNP) patients [86]. A separate observation made by Lecellier *et al.* proposed that PFV was inhibited by cellular miRNA miR-32 [87]. Together, the Bennasser, Omoto and Lecellier reports suggest that primate retroviruses, particularly HIV-1, do indeed interact with the cellular miRNA/RNAi pathways.

### **2.2.3 Suppressors of RNA silencing**

In order to neutralize the inhibitory effects of a putative viral siRNA, Bennasser *et al.* proposed that HIV-1 utilizes its transcriptional transactivating protein, Tat, as an SRS through the direct inhibition of Dicer [85]. Parallel to this, it was also proposed that PFV counters inhibition by cellular miR-32 through the SRS properties of its transcriptional transactivating protein, Tas, although no mechanism was proposed [87].

Many plant and invertebrate viruses encode SRS proteins that operate via a wide variety of mechanisms. In this way, SRS proteins resemble the wide array of interferon inhibitors encoded by many mammalian viruses.

Examples of SRS proteins include the p19 protein of the tomato bushy stunt virus which inhibits RNAi through the direct binding of ~21 nt dsRNAs with 2 nt, 3'

overhangs, preventing the siRNAs from being loaded into RISC [88, 89]. The FHV B2 protein inhibits RNAi through the binding of dsRNAs, regardless of their length, thus preventing Dicer processing [47]. In contrast, the 2b protein of the cucumber mosaic cucumovirus inhibits the production of RdRp-derived viral siRNAs, thereby inhibiting the amplification and spread of the antiviral RNAi effect [90].

## **2.3 Materials and methods**

### **2.3.1 Cell culture and RNA preparation**

293T, HeLa, and MT-2 cells were maintained in DMEM supplemented with 10% FBS. ACH-2 cells were maintained in RPMI 1640 supplemented with 10% FBS and 10 mM HEPES. Total RNA was prepared with TRIzol (Invitrogen) according to manufacture directions. Where appropriate, ACH-2 cells were induced with 1  $\mu$ M PMA (Sigma) for 24 hrs prior to harvest. Levels of p24 production were assessed by HIV-1 p24 ELISA (PerkinElmer), according to the manufacture directions.

Peripheral blood mononuclear cells (PBMCs) were prepared and infected as described in Tomaras *et al.* [91]. Briefly, PBMCs were activated for 3 days with OKT3 and anti-CD28 antibodies, depleted of CD8<sup>+</sup> cells, and allowed 1 day of rest prior to infection. Cells were infected with HIV-1 at a multiplicity of infection of 0.001 using the NL4-3 or QH0515 isolates, or mock infected and then harvested 6 days post-infection. Filtered

supernatants were collected for p24 ELISA, and total RNA was harvested with TRIzol for Northern blot analysis.

293T cells were transduced with pNL-SIN-CMV-BLR as described in Lee *et al.* [92]. 293T cells transduced with pNL-BLR were generated by the same method except that no pcTat was included in the initial transfection. Cells were placed under blasticidin selection 4 days after transduction and cultured at least 21 days total prior to use for experimentation.

### **2.3.2 miRNA cloning**

Cloning of siRNAs and miRNAs out of HTLV-1 and HIV-1 infected cells was conducted as outlined in Lau *et al.* [17] using 750 µg of total RNA from MT-2 or ACH-2 cells, respectively. Identification and classification of sequenced small RNA cDNA clones was based on analysis by GenBank and miRBase databases. The likelihood that we missed potential viral siRNAs in our small RNA cloning was calculated as outlined in Ho *et al.* for zero occurrence situations [93]. Briefly, risk can be estimated with 95% confidence to be no worse than  $3/n$ , where  $n$  is the number of trials.

### **2.3.3 Molecular clones and transfections**

Plasmids pcTat, pK41A, pcTas, pcTax, pcRev, pHIT-G, pcβ-ARR2, pSUPER, pCMV/luc, pSUPER-luc, pBC12/CMV, pBC12/HIV/CAT and pNL-SIN-CMV-BLR have

all been previously described [18, 22, 69, 75, 80, 94-96]. pRL is a *Renilla* luciferase reporter plasmid that was commercially purchased (Promega).

A PCR fragment encoding the 101-amino acid (aa) form of HIV-1 Tat was generated as outlined in Ott *et al.* [97] and cloned into SalI-XhoI digested pcTat to generate pcTat101.

pNL-BLR was derived from pNL-CD4 originally described in Tokunaga *et al.* [98]. The CD4 fragment was removed by XhoI-NotI digestion and replaced with a PCR-generated *blr* fragment.

A full-length TRIM5 $\alpha$  cDNA tagged with a C-terminal HA tag was generated by PCR and cloned into pcDNA3 by AspI-XhoI digestion to generate TRIM5 $\alpha$ -HA.

shTRIM5 $\alpha$  constructs were generated by annealing together primers encoding the entire short hairpin and ligating the double stranded fragments directly into BglII-HindIII digested pSUPER [95]. Three different shTRIM5 $\alpha$  constructs were generated:

shTRIM5 $\alpha$ -1409 (5'-GATCCCCGCTGAAGAATTGGAAGATGACAATATTCATGTCAGCTTCCAACCTTCAGCTTTTTGG-3' and 5'-AGCTCCAAAAGCTGAAGAGTTGGAAGCTGACATGAATATTGTCATCTTCCAATTCTTCAGCGGG-3'), shTRIM5 $\alpha$ -1417 (5'-GATCCCCGCATGCCTCAATGCAAACCTACAAGAATATTCATCTTGTGGTTTGCAGTGAGGCATGCTTTTTG-3' and 5'-AGCTCAAAAAGCATGCCTCACTGCAAACCA CAAGATGAATATTCTTGTAGTTTGCATTGAGGCATGCGGG-3') and shTRIM5 $\alpha$ -1422 (5'-GATCCCCGCCTTACGAATTCTGAAATTGAGATATATTCAATCTCAGTTTCAGA

GTTCGTAAG GCTTTTTG-3' and 5'-AGCTCAAAAAGCCTTACGAACTCTGAAACTG  
AGATTGAATATATCTCAATTTTCAGAATTCGTAAGGCGGG-3').

All transfections were performed using calcium phosphate.

### **2.3.4 Northern blots**

miRNAs were detected by Northern blot as previously described in Cai *et al.* [15] using 30 µg of total RNA. For Northern blots of HTLV-1 and HIV-1 genomic transcripts, 10 µg of total RNA from MT-2 and ACH-2 cells, respectively, were separated on a 0.6% agarose gel. RNA was transferred onto nitrocellulose and fixed by UV irradiation and baking at 80°C under vacuum. Blots were hybridized to probes generated by random priming of BglII-SphI fragments of the HTLV-1 LTR, or KpnI-HindIII fragments of the HIV-1 LTR. Bands were visualized by exposing blots to film at -80°C with intensifying screens.

### **2.3.5 Western blots**

293T cells were transfected with 500 ng of pcTat, pcTas, pcTax or pBC12/CMV, 500 ng of TRIM5α-HA, 500 ng of β-ARR2, and 500 ng total of shTRIM5α (1409, 1417 and 1422) or pSUPER filler. Cells were harvested 48 hrs post-transfection and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Samples were transferred to nitrocellulose and probed with a monoclonal mouse antibody specific for the HA tag

(Covance). Bands were visualized with Lumi-Light Western Blotting Substrate (Roche) according to manufacture directions.

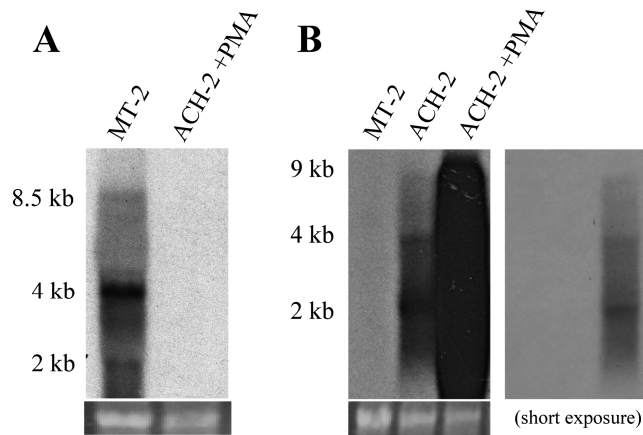
### **2.3.6 Luciferase assays**

HeLa cells were transfected with 3000 ng of pSUPER-luc or pSUPER filler, 100 ng of pCMV/luc, 100 ng of pRL, and various amounts of pcTat101, pcTas or pBC12/CMV as described. Cells were harvested 48 hrs post-transfection and luciferase activity was assayed with Dual-Luciferase Reporter Assay System (Promega) according to manufacture directions.

## **2.4 Results**

Small RNAs were cloned out of MT-2 and ACH-2 cells persistently infected with HTLV-1 and HIV-1, respectively. MT-2 cells are derived from human T-cells isolated from cord blood lymphocytes co-cultured with cells from HTLV-1 infected patients [99]. The cells contain at least one intact copy of a provirus integrated into the host genome and continuously shed infectious HTLV-1 particles [100]. Northern blot analysis of total RNA harvested from MT-2 cells using a probe specific for the HTLV-1 LTR demonstrated that the full-length (~8.5 kb), singly-spliced (~4 kb), and doubly-spliced (~2 kb) transcripts were all being produced (Fig. 2-4A).

ACH-2 cells are derived from CEM, a human T-cell line infected with the LAV strain of HIV-1; ACH-2 was a clone that survived the initial infection [101]. The cells continuously produce low levels of infectious virus, and treatment with phorbol myristate acetate (PMA) induces high level virus production followed by cell death. HIV-1 p24 ELISA determined that uninduced cells produce ~4.1 ng of capsid protein per mL of supernatant per day. After PMA induction, the level of p24 production increased



**Figure 2-4. MT-2 and ACH-2 express readily detectable amounts of viral transcripts.** Northern blot analysis of MT-2, uninduced ACH-2 and PMA-induced ACH-2 cells. Blots were hybridized to probes specific for the viral LTRs to demonstrate that all viral transcripts (i.e., full-length, singly-spliced and doubly-spliced variants) were produced. A) MT-2 cells producing HTLV-1 transcripts. B) ACH-2 cells producing HIV-1 transcripts. Two separate exposures are provided to demonstrate the potent effect of PMA-induction on HIV-1 transcription. Ethidium bromide staining of rRNA bands serve as loading controls.

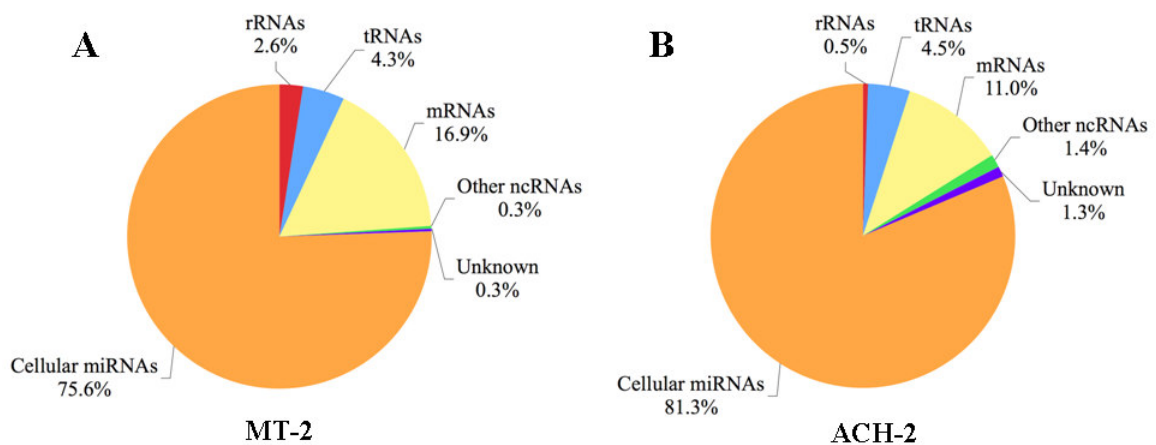
to ~146 ng per mL per day. Northern blot analysis of total RNA isolated from both induced and uninduced cells using a probe specific for the HIV-1 LTR demonstrated that all viral transcripts including the full-length (~9 kb), singly-spliced (~4 kb) and doubly-spliced (~2 kb) species were being produced (Fig. 2-4B).

Small RNAs 18-24 nts in size were harvested from MT-2 and ACH-2 cells for cDNA cloning. The resultant sequences were identified and categorized based on comparison to the GenBank and miRBase databases. Analysis of 698 MT-2-derived cDNAs revealed that the vast majority (75.6%) were cellular miRNAs, as expected (Fig. 2-5A). The remaining cDNAs were identified as degradation products of mRNAs (16.9%), tRNAs (4.3%), and rRNAs (2.6%). Of all the sequences examined, none were of HTLV-1 origin.

Analysis of 625 ACH-2-derived cDNAs gave similar results (Fig. 2-5B). The vast majority of cDNAs were again identified as cellular miRNAs (81.3%) while the remaining sequences were identified as breakdown products from mRNAs (11.0%), tRNAs (4.5%) and rRNAs (0.5%). As with HTLV-1, no cDNAs of HIV-1 origin were recovered.

The small RNA cloning protocol we used only captures RNAs with a 5' monophosphate moiety, characteristic of RNase III cleavage by enzymes such as Drosha and Dicer [21]. To verify that small viral RNAs were not being generated by a Drosha- or Dicer-independent mechanism, cloning was repeated on the same small RNA pool with

an alternative protocol that captures all RNAs regardless of 5' phosphorylation state. Although this method recovers a wider pool of potential targets, it is not typically used because of the high percentage of breakdown products that contaminate the final cDNA population (e.g., 5'-unphosphorylated, -diphosphorylated and -triphosphorylated degradation products are all recovered). Sequencing of 18-24 nt cDNAs generated via this protocol recovered a high percentage of mRNA break-down products and cellular miRNAs, as expected, but again, no small RNAs of viral origin (data not shown).

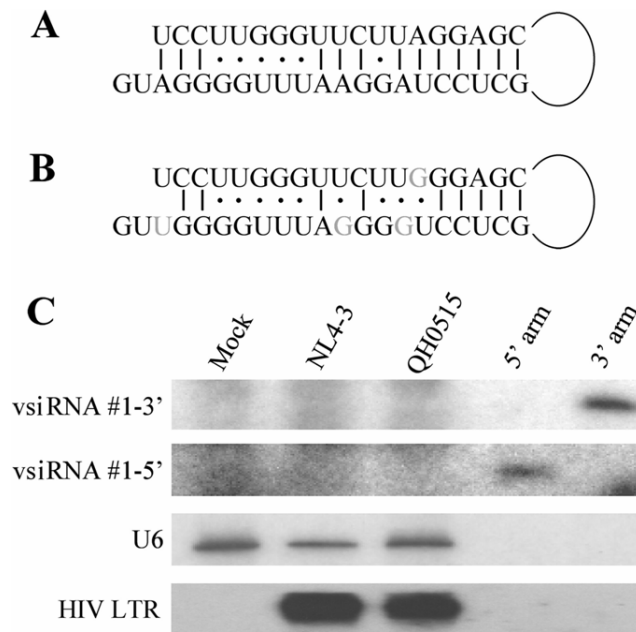


**Figure 2-5. Summary of small RNAs recovered from MT-2 and ACH-2 cells.** RNAs 18-24 nts were harvested from MT-2 and ACH-2 cells for cDNA cloning. A total of 698 MT-2- and 625 ACH-2-derived cDNAs were sequenced and identified by reference to GenBank and miRBase. No small RNAs of viral origin were identified in either sample.

Together these data suggest that HTLV-1 and HIV-1 neither encode miRNAs nor generate siRNAs during infection. We note that both the MT-2 and ACH-2 cell lines used for our cloning experiments continuously shed infectious virus and produce all expected viral transcripts including the full-length species. Therefore, all potential substrates for Drosha/Dicer cleavage are present and available for processing. In spite of this, we were unable to recover any small RNAs of viral origin from either sample using two different cloning protocols. Although it is impossible to completely rule out the existence of any HTLV-1 or HIV-1 siRNAs or miRNAs, these data argue that should they exist, these small RNAs must be present at extremely low levels. Based on our data, we calculate that they would constitute < 0.5% of all Dicer-generated small RNAs from infected cells ( $P < 0.05$ ).

Bennasser *et al.* previously proposed that a perfect stem-loop hairpin located within the HIV-1 *env* gene is processed by Dicer into an siRNA which they designated vsiRNA#1 [85]. They were purportedly able to detect this siRNA by Northern blot of T-cells infected with NL4-3, a laboratory strain of HIV-1 closely related to the LAV strain found in ACH-2 cells. It should be noted however, that the proposed stem-loop structure from which vsiRNA#1 is derived, as diagramed in the paper, actually represents a computationally predicted sequence based on a survey of 721 sequences in the Los Alamos HIV-1 *env* database (Fig. 2-6A). Notably, some of the nucleotide choices required to make the perfect stem of this theoretical hairpin occur very infrequently; two

nucleotides occur < 5%. More importantly, an HIV-1 strain encoding the actual diagrammed sequence does not exist in the Los Alamos database. In addition, although not explicitly diagrammed, the vsiRNA#1 terminal loop is 197 nts—far larger than typical miRNA terminal loops which are usually only 10 to 17 nts long [102].



**Figure 2-6. HIV-1-infected PBMCs fail to express detectable levels of vsiRNA#1.**

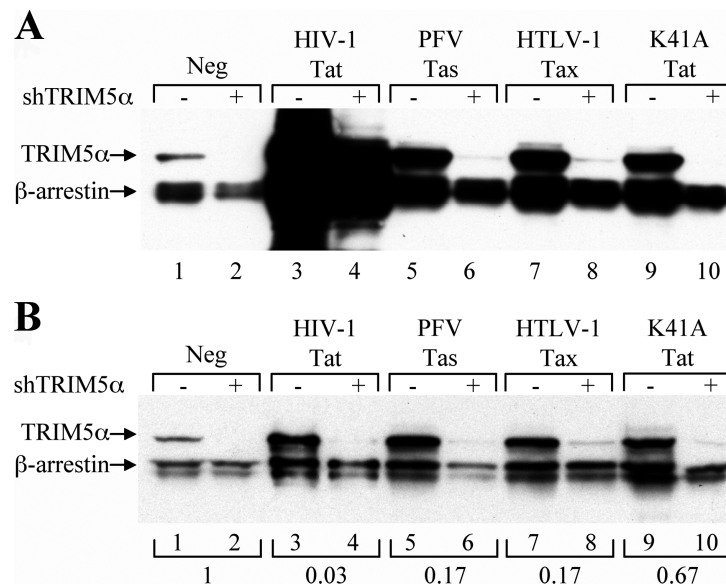
A) Secondary structure of proposed vsiRNA#1 stem-loop as diagrammed in Bennisser *et al.* [85]. B) Secondary structure of NL4-3 folded into proposed vsiRNA#1 stem-loop. Differences in sequence are indicated by grey nucleotides. C) Northern blot for the 3' and 5' arms of vsiRNA#1 in mock-, NL4-3- or QH0515-infected PBMCs. Synthetic oligonucleotides identical to vsiRNA#1 served as positive controls and were loaded onto the blot at a concentration of ~300 copies per cell. The same blot was hybridized to an HIV-1 LTR-specific probe to demonstrate robust expression of viral transcripts. U6 serves as a loading control.

Moreover, the secondary structure of this entire locus has already been verified both *in vivo* and *in vitro* [103, 104] to exist in an alternative configuration dramatically different than that proposed by Bennasser *et al.*; vsiRNA#1 is entirely contained within the RRE, a complex secondary structure absolutely required for Rev binding and subsequent export of viral transcripts from the nucleus [76, 103]. Without the RRE, all structural genes would be spliced out of the viral mRNAs and HIV-1 infection would be totally inhibited. Moreover, alignment of vsiRNA#1 with the actual NL4-3 sequence demonstrates that differences between the two sequences would make it very difficult for NL4-3 to assume the proposed configuration based on the low binding energy of the stem (Fig. 2-6B).

Nevertheless, in an effort to determine if vsiRNA#1 could actually be detected in HIV-1-infected cells, RNA was harvested from peripheral blood mononuclear cells (PBMCs) infected with NL4-3 or QH0515, a primary HIV-1 isolate [105], for Northern blot analysis. Also included on the blot was a synthetic RNA oligonucleotide identical to vsiRNA#1 loaded at a concentration equivalent to ~300 copies per cell. Hybridization with a probe specific for the HIV-1 LTR demonstrated that there was robust infection of the PBMCs, and ample amounts of viral transcripts were being produced and available for Dicer processing. In spite of this, neither arm of vsiRNA#1 could be detected (Fig. 2-6C). Although these data do not definitively rule out the existence of the

Bennasser HIV-1 siRNAs, it does suggest that, should they exist, they must be present at exceedingly low levels.

To determine if the HIV-1 transcriptional transactivator, Tat, has SRS properties, co-transfection experiments were performed with a previously validated shRNA system. shTRIM5 $\alpha$  is an RNA pol III-driven expression construct that generates a TRIM5 $\alpha$



**Figure 2-7. Retroviral transactivators do not inhibit RNAi in 293T cells.** Cells were co-transfected with a short-hairpin construct targeting TRIM5 $\alpha$  (shTRIM5 $\alpha$ ) as well as HA-tagged forms of TRIM5 $\alpha$  and  $\beta$ -arrestin, in the presence of various retroviral transactivators. A) Expression of shTRIM5 $\alpha$  is able to inhibit TRIM5 $\alpha$  expression while leaving  $\beta$ -arrestin levels unchanged (lanes 1 and 2). However, the presence of the transactivators non-specifically increases expression of both proteins (lanes 3 to 10). B) Samples from panel A have been normalized according to the amounts indicated at the bottom of the panel to compensate for non-specific transactivation effects.

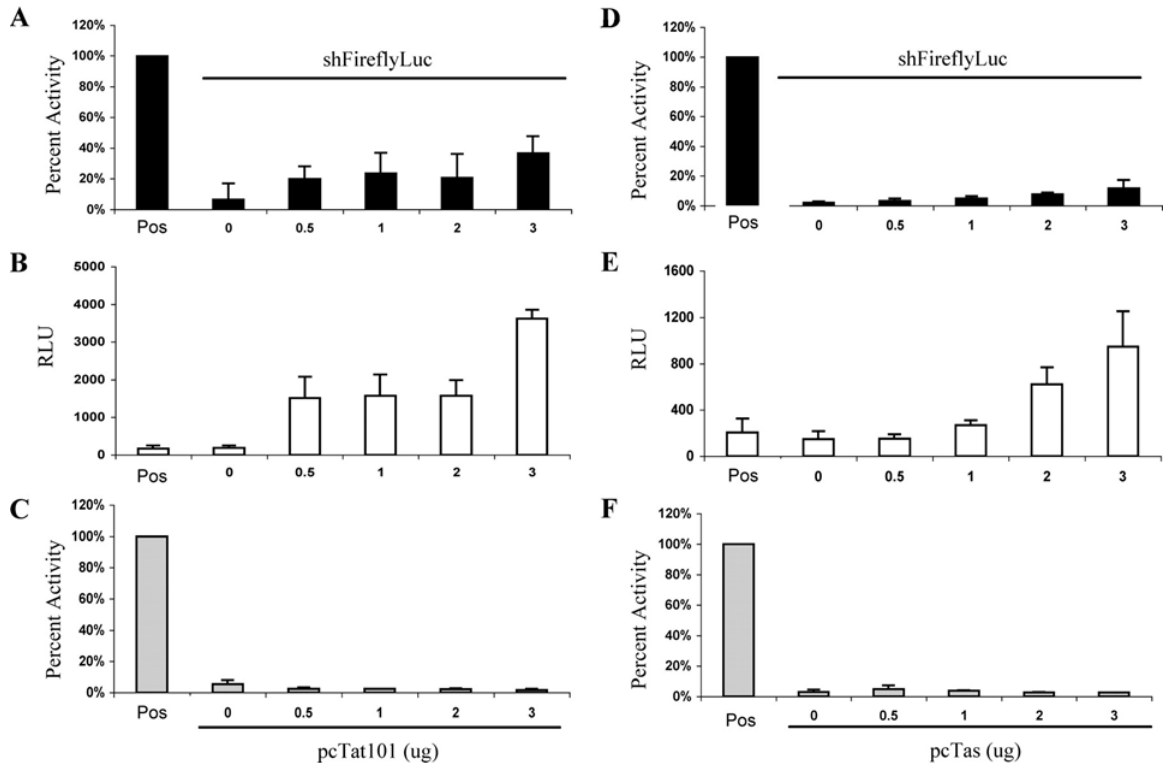
shRNA processed by Dicer [95]. The resultant siRNAs are capable of potentially inhibiting expression of HA-tagged TRIM5 $\alpha$  during co-transfection (Fig. 2-7A, compare lane 1 to lane 2). A plasmid expressing an HA-tagged  $\beta$ -arrestin protein was also included as an internal control. If Tat acts as an SRS through direct inhibition of Dicer as proposed by Bennasser *et al.* [85], then Tat expression should block the processing of shTRIM5 $\alpha$  into mature siRNAs and inhibit TRIM5 $\alpha$  knock-down.

Co-transfection of shTRIM5 $\alpha$  and various retroviral transactivators including HIV-1 Tat, PFV Tas, and HTLV-1 Tax into 293T cells demonstrated that all transactivators non-specifically increased expression levels of both HA-TRIM5 $\alpha$  and HA- $\beta$ -arrestin (Fig. 2-7A, lanes 3-8). This was not unexpected as it has been previously reported that Tat, Tas and Tax all are capable of activating heterologous promoters when highly expressed [83, 106-108]. When the results are normalized for this non-specific activity, however, it is clear that these transactivators are incapable of inhibiting RNAi (Fig. 2-7B, lanes 3-8).

Bennasser *et al.* also reported that Tat K41A [109], a transcriptionally inactive Tat mutant unable to bind its cellular co-factor cyclin T1, still retained an SRS phenotype [85]. When we assessed Tat K41A in our co-transfection assays, we found that it was indeed transcriptionally deficient compared to wild type Tat (Fig. 2-7A, compare lanes 3 and 4 to lanes 9 and 10). However, similar to wild-type Tat, K41A was unable to inhibit shTRIM5 $\alpha$ -mediated knock-down of HA-TRIM5 $\alpha$  (Fig. 2-7B, lanes 9 and 10).

Although our western blot data disagree with findings reported by Bennasser *et al.* [85], we note two differences between our experiments and theirs; we performed our experiments in 293T cells using an 86-aa form of Tat while Bennasser used HeLa cells and a 101-aa form of Tat. The 86-aa form of Tat is fully functional as a transactivator of the HIV-1 LTR [110] and is found in many laboratory strains of HIV-1 including NL4-3, LAV, HXB-2, and even in some primary isolates.

In order to demonstrate that our findings were not the result of cell line- and/or Tat-specific artifacts, we conducted a series of luciferase experiments designed to mimic those published by Bennasser *et al.* [85]. Co-transfection experiments using shLuc to inhibit expression of firefly luciferase was performed in HeLa cells. Although shLuc is able to reduce firefly luciferase activity by ~10-fold, activity appears to be partially rescued by co-transfecting increasing amounts of 101-aa Tat (Fig. 2-8A). Analysis of the same samples for renilla luciferase activity, however, demonstrates that this apparent rescue was due to non-specific transactivation by Tat [107, 108]; even though equal amounts of renilla luciferase plasmid were co-transfected with each sample as an internal control, renilla activity increases concordant with the amount of Tat in each sample (Fig. 2-8B). (Note that renilla activity in the two samples without Tat is approximately equal.) Once firefly luciferase activity is normalized with the renilla data, it is clear that Tat does not inhibit RNAi regardless of the amount co-transfected



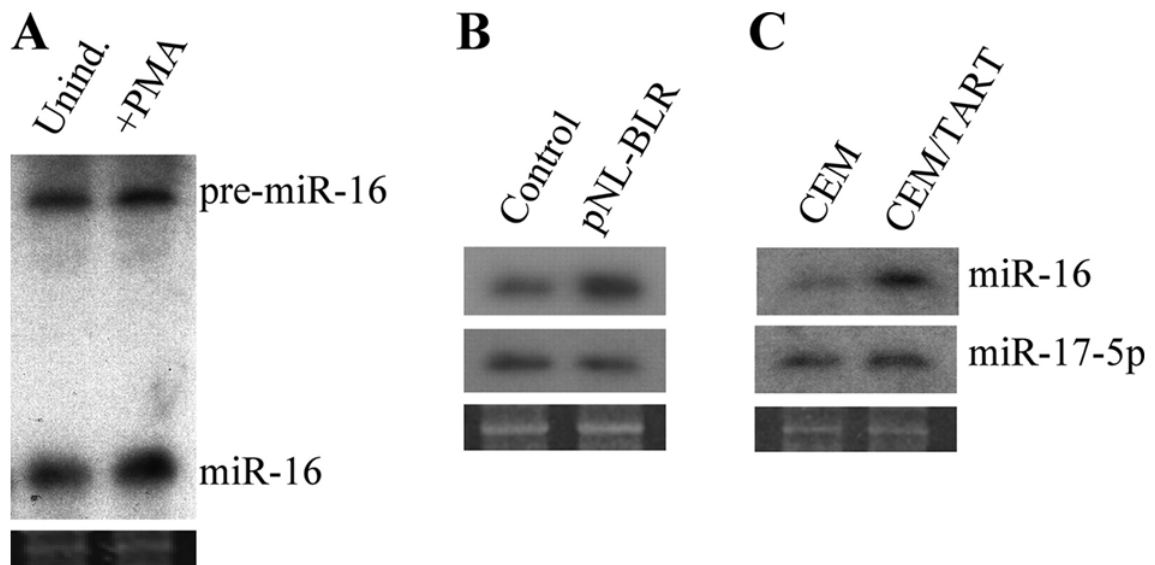
**Figure 2-8. HIV-1 Tat and PFV Tas do not inhibit RNAi in HeLa cells.** Cells were co-transfected with FLuc, RLuc, a short-hairpin expressing FLuc-specific siRNAs (shFireflyLuc) and indicated amounts of either 101-aa Tat (pcTat101) (panels A to C) or Tas (pcTas) (panels D to F). Panels A and D, FLuc activity levels, in the presence and absence of shFireflyLuc (Pos). Values obtained in the absence of shFireflyLuc were set to 100% and all other values normalized accordingly. Panels B and E, internal control RLuc values of the same samples assayed in panels A and D. Panels C and F, FLuc activity of panels A and D after normalization with RLuc values of panels B and E. RLU, relative light units.

(Fig. 2-8C). The same series of experiments were also conducted with PFV Tas (Fig. 2-8D, E and F). Although the level of non-specific transactivation by Tas is less dramatic than that observed for Tat, it is also clear that Tas does not inhibit RNAi regardless of the amount co-transfected.

HIV-1 Tat was also proposed to act as an SRS through the direct inhibition of Dicer [85]. A consequence of this theory would be the inhibition of mature miRNA production in the presence of Tat. To test this, the effect of Tat on cellular miRNA biogenesis was examined by Northern blot analysis. ACH-2 cells were chemically induced with PMA for 24 hrs resulting in an ~40-fold increase in levels of HIV-1 p24 (Fig. 2-4B). RNA from both induced and uninduced cells were harvested, and Northern blot analysis demonstrated that levels of cellular miRNA miR-16 remained unchanged in both samples and did not decline in the presence of increased Tat expression (Fig. 2-9A).

To extend this data, we examined if *de novo* expression of Tat at physiological levels has an effect on miRNA biogenesis. 293T cells were transduced with pNL-BLR, a non-replicating HIV-1 vector deleted for the *vpr*, *rev* and *env* genes with *blr* replacing *nef*. The vector does contain functional copies of the five remaining HIV-1 genes and produces pseudotyped virus when co-transfected with plasmids expressing Rev and the VSV glycoprotein. Viruses were harvested and used to transduce fresh 293T cells which were then placed under blastocidin selection. After infection, the only gene products

produced by the vector are Blr and Tat; the lack of Rev prevents any gene encoded on either the full-length or singly-spliced transcripts from being expressed. Since expression of both Blr and Tat are driven by the HIV-1 LTR promoter, they are expressed at physiological levels. As a control, a parallel set of 293T cells were transduced with a similar vector, pNL-SIN-CMV-BLR, which produces Blr, but no Tat [92]. RNA from both samples was harvested for Northern blot and analysis of cellular miRNAs miR-16 and



**Figure 2-9. Tat does not inhibit cellular miRNA biogenesis.** A) Northern blot analysis of cellular miR-16 expression in both uninduced ACH-2 and PMA-induced cells. B) Northern blot analysis of cellular miR-16 and miR-17-5p levels in 293T cells transduced with pNL-SIN-CMV-BLR expressing Blr, compared to cells transduced with pNL-BLR expressing both Tat and Blr. C) Northern blot analysis of cellular miR-16 and miR-17-5p levels in untransduced CEM cells, compared to cells transduced with TART, which express Tat and Rev. Ethidium bromide staining of rRNAs serve as loading controls.

miR-17-5p demonstrated that levels of neither miRNA decreased in the presence of Tat (Fig. 2-9B).

To extend this data to a more relevant cell line, we also examined a CEM T-cell line transduced with a vector, TART, which expresses both Tat and Rev [111]. Expression of Tat and Rev in CEM/TART cells is driven by the HIV-1 LTR promoter which, again, provides expression of both proteins at physiological levels. Transfection of CEM/TART cells with an HIV-1 LTR-driven luciferase reporter verified Tat expression (data not shown). RNA from CEM/TART cells and untransduced CEM cells were harvested for Northern blot analysis which demonstrated that levels of cellular miR-16 and miR-17-5p were, again, not affected by Tat expression (Fig. 2-9C).

## **2.5 Discussion**

The question of whether retroviruses interact with the cellular miRNA/RNAi machinery during infection of mammalian cells has been controversial [84]. Several papers recently addressing this issue have come to conflicting conclusions.

The most comprehensive examination of this issue performed to date was conducted by Pfeffer *et al.* [57]. The authors cloned small RNAs from various cell lines infected with a number of herpesviruses including Kaposi's sarcoma-associated virus (KSHV), mouse gamma-herpesvirus 68 (MHV68), and human cytomegalovirus (HCMV). For comparison, small RNAs were also cloned from cell lines infected by three

different small-RNA viruses—HIV-1, YFV, or HCV. Despite being able to recover hundreds of virally-derived cDNAs from the herpesvirus samples which were later determined to be novel viral miRNAs, no miRNAs or siRNA of viral origin were recovered from samples infected with HIV-1, YFV or HCV.

In direct contradiction, Bennasser *et al.* [85] and Omoto *et al.* [86] reported the existence of an HIV-1 siRNA and miRNA, respectively. In a separate publication, Lecellier *et al.* also published a report demonstrating that PFV was inhibited by cellular miRNA miR-32 [87].

After cDNA cloning of 18-24 nt RNAs from persistently infected MT-2 and ACH-2 cells, we were unable to recover any miRNAs or siRNAs of viral origin (Fig. 2-5) leading us to the same conclusion as Pfeffer *et al.* [57]. Unlike all other RNA viruses, retroviruses do not produce long, perfectly complementary dsRNAs at any point during their life cycle. Although they have relatively small regions of complementarity within their genomes in the form of imperfect hairpins (e.g., TAR or the RRE), they are, on the whole, sub-optimal substrates for the generation of siRNAs. Moreover, it is well established that in mammalian cells the presence of perfectly complementary dsRNAs, even those as short as ~21 nts, triggers the interferon system, a complex cascade of cellular events resulting in global inhibition of cellular translation [112]. Given this, we would not expect to find siRNAs of retroviral origin in mammalian cells.

The lack of potential HIV-1 miRNAs recovered is also an unsurprising result. Since Drosha cleavage of the pri-miRNA would effectively destroy the viral transcript [15], any miRNAs produced must be critical for the HIV-1 life cycle in order to justify the destruction of viral mRNAs. The proposed Omoto miRNA [86], however, is not well conserved among the various HIV-1 subgroups which suggests that it does not play an important role in HIV-1 replication (data not shown). As the Omoto miRNA was not recovered from two separate cloning efforts, we conclude that it must be expressed at very low levels, if it exists at all.

Although the cDNA sequencing data is informative in determining whether or not viral siRNAs or miRNAs are generated during infection, it cannot tell us whether PFV is inhibited by a cellular miRNA as proposed by Lecellier *et al.* [87]. The authors demonstrated that reporter constructs expressing portions of the PFV genome fused to GFP were down-regulated by miR-32 when transfected into 293T cells, a cell line of human origin. Therefore, the authors proposed that the cellular miRNA serves as an antiviral defense against PFV infection. However, since miR-32 is highly conserved across a wide variety of vertebrates including opossums, wild boars, chickens and humans [12, 13], it seems unlikely that the miRNA was specifically evolved to combat PFV, a chimpanzee-specific virus [113]. It should also be noted that the portion of PFV targeted by miR-32 is not well conserved among other foamy viruses (data not shown), suggesting that this sequence is not of evolutionary importance. Therefore, although the

observations made by Lecellier *et al.* may indeed be accurate, they are most likely an artifact of the cell line used.

We also demonstrate here that HIV-1 Tat does not exhibit SRS properties (Figs. 2-7 and 2-8). The inability of Tat to inhibit Dicer in two different cell lines, using two different versions of Tat, with two different shRNA reporter systems, suggests that these results are indeed genuine. Less exhaustive analysis of PFV Tas and HTLV-1 Tax suggests that they also do not act as SRS proteins. However, it was clear in all of our experiments that although the natural target of these proteins are the LTR promoters of their cognate viruses, all are potent activators of heterologous promoters when expressed at high levels as previously reported [83, 106-108]. This non-specific transactivation effect could account for the perceived rescue of RNAi reported by both Bennasser *et al.* and Lecellier *et al.* [85, 87].

Through Northern blot analysis we also demonstrated that Tat neither directly nor indirectly inhibits Dicer cleavage; levels of cellular miRNAs miR-16 and miR-17-5p did not decline in response to Tat expression (Fig. 2-9). The most compelling piece of evidence is Fig. 2-9C where Tat was expressed at physiological levels in a relevant T-cell line. The CEM/TART cells have been in existence for at least several months which is more than enough time for the entire miRNA population to turnover. As such, this system should have allowed us to detect any inhibitory effects Tat may have had on miRNA biogenesis. Analysis of miR-16 and miR-17-5p, however, indicated that levels of

neither miRNA decreased in the presence of Tat. (Interestingly, miR-16 levels actually seem to increase.) Moreover, it is difficult to imagine a mechanism by which Tat, a nuclear transcription factor [71], could directly inhibit Dicer, a cytoplasmic protein [63] as proposed by Bennasser *et al.* [85].

As a whole, we find our results to be in good agreement; it is not necessary for Tat to have SRS properties if HIV-1 does not generate antiviral siRNAs during infection. Our results suggest that this conclusion also applies the *retroviridae* family in general, and not simply just to HIV-1. Therefore, although RNAi is a formidable antiviral defense mechanism for plants and invertebrates [46, 47], it does not appear to play a role in the mammalian response to retroviral infection.

## 3. Herpes simplex virus 1 encoded microRNAs

### 3.1 Summary

During latency, herpes simplex virus 1 (HSV-1) produces only one transcript in abundance—the latency-associated transcript (LAT). Although this transcript is capped and polyadenylated, the LAT is highly unstable and the full-length form is rarely detected. Several lines of evidence have suggested that this instability is due to LAT functioning as a pri-miRNA precursor that is being rapidly cleaved and processed into mature miRNAs. Through deep sequencing of both LAT-transfected cells and latently-infected mouse trigeminal ganglia (TG), we were able to identify five miRNAs encoded by HSV-1. These miRNAs were all found to be expressed during latency and contained within LAT except for one, miR-H6, which is derived from the opposite strand, ~450 bp upstream of the LAT transcription start site. Mapping of the LAT miRNAs onto the HSV-1 genome determined that one, miR-H2-3p, is located directly antisense to *ICP0*, an important transcriptional activator involved in reactivation of the virus from latency. Through western blot analysis we determined that miR-H2-3p is capable of potently inhibiting ICP0 protein expression, but despite perfect complementarity, surprisingly, does not cleave or degrade the target mRNA. Computational analysis also identified a high affinity match between the seed region of miR-H6 with *ICP4*, another viral transcriptional activator required for HSV-1 replication.

ICP4 may also play a role in reactivation of HSV-1 from latency, and previous work has demonstrated that its expression is post-transcriptionally regulated by LAT. Protein and mRNA analysis indicate that miR-H6 acts through a mechanism of translational inhibition, potentially down-regulating ICP4 protein expression while leaving *ICP4* mRNA levels essentially unchanged.

## **3.2 Introduction**

### **3.2.1 Herpesviruses**

Herpesviruses are large, complex double-stranded DNA viruses (reviewed in [114]). The linear genome is contained within a capsid, and the entire virion is surrounded by a glycoprotein envelope. They are a highly disseminated family of viruses and nearly all animal species examined to date have been found to host at least one variety of herpesvirus.

The most notable characteristic of herpesviruses is their ability to establish a lifelong latent infection in the natural host [114]. During latency, no infectious progeny are produced and only a small subset of viral genes is actively transcribed. Viruses may periodically reactivate from the latent state to undergo productive replication at the original site of infection. The molecular mechanisms involved in establishing and

maintaining latency, as well as the reactivation event itself, however, remain poorly understood.

The *herpesviridae* family is divided into alpha-, beta- and gamma-subfamilies based on sequence homology, genome architecture, host range, replication kinetics, and preferred cell type for latent infection [114]. Alpha-herpesviruses are characterized by short reproductive cycles and a preference for establishing latency in sensory ganglia. Beta-herpesviruses tend to have a restricted host range and long, slow infectious cycles. Latency may be established in a variety of tissues including secretory glands, lymphatic and myeloid cells. In contrast, gamma-herpesviruses typically replicate in lymphocytes and endothelial cells and establish latency in lymphoid and endothelial tissues.

### **3.2.2 Herpesvirus miRNAs**

Herpesviruses are ideally suited to encode miRNAs—they are nuclear, DNA viruses that establish latent infections maintained for the life of the host. In fact, every herpesvirus examined to date has been shown to encode miRNAs, including several human herpesviruses of the beta- and gamma-subfamilies including human cytomegalovirus (HCMV), Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) [57-59]. Expression analysis has determined that while some miRNAs are expressed during latency, others are expressed during lytic replication, suggesting that they may have stage-specific functions in the virus life cycle [61]. There

is considerable interest in identifying the targets of these miRNAs in order to further elucidate the interaction between herpesviruses and their host cells.

In 2006 Cui *et al.* reported the discovery of the first miRNA encoded by an alpha-herpesvirus [115]. This miRNA was designated miR-H1, and is expressed as a late gene during productive infection by HSV-1.

### **3.2.3 Regulation of viral protein expression by viral miRNAs**

Thus far there have been four reported examples of viral protein regulation via the actions of viral miRNAs. The first to be described was EBV miR-BART2 regulation of the viral DNA polymerase gene, *BALF5* [116]. As miR-BART2 is located antisense to *BALF5*, the miRNA is perfectly complementary and acts as an siRNA by cleaving the target transcript precisely 10 nts downstream from the 5' end of the miRNA binding site. (Interestingly, this *BALF5* cleavage product was first mapped and reported more than a decade ago in 1993 [117], well before the discovery of the EBV miRNAs.) Since miR-BART2 is typically expressed during latency, it is hypothesized that this miRNA suppresses *BALF5* production in order to maintain an effective latent state.

The second case involves the SV40 miRNA miR-S1, which is also located antisense to its target, the viral T antigen [54]. As a result, expression of miR-S1 in infected cells down-regulates T antigen protein production through cleavage and degradation of the viral mRNA. Although expression of the miRNA does not affect

virus replication *per se*, down-regulation of the viral T antigen does reduce susceptibility of SV40-infected cells to cytotoxic T-cell killing.

A separate report on EBV miRNAs demonstrated that at least three miRNAs located within the EBV BART Cluster 1 all target the 3'UTR of the viral *LMP1* mRNA [118]. *LMP1* has tumorigenic properties through its ability to activate a number of cellular pathways including JAK/STAT, NFκB, and Ras/MAPK, and has been implicated as the driving force behind several EBV-associated cancers [119]. Paradoxically, however, *LMP1* over-expression can inhibit cell growth and sensitize cells to apoptosis. Therefore, EBV must carefully modulate *LMP1* levels in order to establish an optimal environment for viral replication. The authors propose that this regulation is accomplished through the actions of the Cluster 1 BART miRNAs.

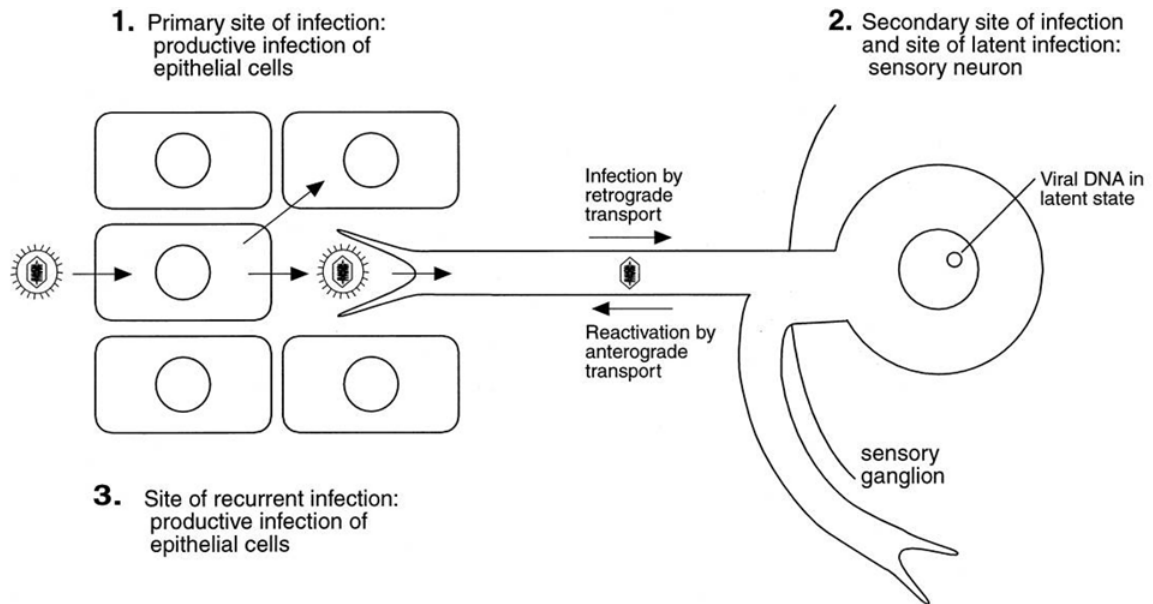
The most recent report involves miRNA miR-UL112-1 encoded by HCMV. Grey *et al.* demonstrated that miR-UL112-1 is able to target the 3'UTR of three different viral proteins including IE gene *IE72*, as well as *UL112* and *UL120* [60]. *IE72* was a particularly interesting target since it is an immediate-early gene required for the initiation of productive replication. During viral infection, *IE72* is initially expressed at high levels. As infection progresses, however, protein levels decline as levels of miR-UL112-1 increase. When miR-UL112-1 was prematurely expressed prior to HCMV infection, a dramatic decrease in viral DNA genome levels was detected, suggesting that miR-UL112-1 plays a role in viral replication.

These four reports demonstrate that viral miRNA regulation of viral gene expression is a common strategy employed by many viruses, and that this regulation may be accomplished via either an siRNA- or miRNA-mediated mechanism of inhibition.

In addition to the “auto-regulation” of viral transcripts, it is also possible that viral miRNAs target cellular genes. In fact, it has been proposed that the majority of viral miRNAs do not play a direct role in viral replication, but instead serve to inhibit the antiviral response initiated by infected cells [120]. Several examples of this have already been described [36, 55, 56] (including the SV40 miR-S1 down-regulation of the viral T antigen [54]).

### **3.2.4 Herpes simplex virus, type 1**

HSV-1 is the prototypical member of the alpha-herpesvirus subfamily; it was one of the first human herpesviruses ever isolated over 40 years ago and remains one of the most well-studied viruses (reviewed in [121]). The initial site of infection is typically in epithelial cells at the mucosal membrane of either the eyes, nose or mouth (Fig. 3-1). Following productive infection, viruses travel via intra-axonal retrograde transport to neurons of the TG where latency is established for the life of the host [122, 123]. HSV-1 is capable of sporadic reactivation where productive replication is observed at, or near, the original site of infection. During reactivation when new viral progeny are



**Figure 3-1. HSV-1 life cycle.** 1) The initial round of productive infection occurs in the epithelial cells of the mucosal membrane. During this time, infectious progeny are produced and the virus is capable of spreading to new hosts and other cells near the initial infection site. 2) Viruses travel by retrograde intra-axonal transport through sensory neurons to the TG. There is a brief period of acute replication lasting approximately one week after which the virus becomes fully latent. During latency, the viral genome exists as a circular episome in the host cell nucleus and the only viral gene product abundantly expressed is LAT. 3) Stimuli such as UV light, temperature change or stress reactivate the virus, resulting in a new round of viral production. Newly synthesized progeny travel back through the sensory neurons to the original site of infection where a new round of productive replication is initiated. Latency is eventually re-established and this cycle is periodically repeated for the life of the host. (Figure adapted from [121].)

produced, the virus is infectious and may be spread to new hosts. Although reactivation may be triggered by a variety of stimuli including UV light, temperature changes and stress, the molecular mechanism underlying the switch is poorly understood. Humans are the only natural hosts of HSV-1.

The linear genome of HSV-1 contains two unique sequence domains—the unique long ( $U_L$ ) and unique short ( $U_S$ ) regions [124], each flanked by their own set of inverted repeats [125, 126]. Depending on their location in the genome, these repeats are designated as either terminal repeats (TR) or internal repeats (IR). They are further distinguished by which unique region they flank (e.g., repeats flanking the  $U_L$  region are designated as  $TR_L$  and  $IR_L$ ).

During productive HSV-1 infection, more than 80 gene products are expressed in a highly-regulated cascade that begins with the immediate-early genes (IE), progresses to the early genes (E), and finally through to the late genes (L) [127-129]. These genes produce proteins involved in viral gene expression, viral DNA replication, and virion assembly, respectively. All viral transcripts are generated in the nucleus by the host cell RNA pol II, after which they are exported to the cytoplasm for translation.

Expression of the IE genes begins as early as one hour post-infection [130] independent of *de novo* synthesis of other viral proteins. The IE gene family includes only six proteins – ICP0, ICP4, ICP22, ICP27, ICP47 and  $U_S1.5$ . Except for ICP47, these

proteins facilitate progression of the virus from the IE to the E phase by initiating transcription of the E genes [129].

Unlike the majority of E and L genes which are encoded within the unique regions, most IE genes (i.e., ICP0, ICP4, ICP22 and Us1.5) are located within the repeat sequences of the genome [124, 125, 131]. Therefore, once the viral genome circularizes upon entry into the nucleus [132], there are two copies per genome of every gene contained within the repeats. As a consequence, these genes produce twice as much protein as those encoded only once. This seems to be significant in that, at least for ICP0, viruses encoding only one copy of the gene are severely impaired in their ability to reactivate from latency [133].

ICP0 is the first gene to be expressed during infection [134] and is capable of potently up-regulating transcription of all three classes of viral genes [135-137]. It has not been possible to determine a consensus ICP0 binding site in activated genes [138] which suggested that the protein acts as a transcriptional activator through an indirect mechanism, rather than direct DNA binding. This hypothesis was supported by the observation that ICP0 encodes a RING finger domain similar to those found in other members of the ubiquitin ligase family [139, 140], and the fact that ICP0 requires an active proteasome pathway for full stimulatory activity [141, 142]. Subsequent studies determined that ICP0 promotes the selective translation of viral transcripts through the destabilization of cellular mRNAs via the ubiquitin degradation pathway [142-144].

In cell culture, ICP0-null viruses are replication-impaired at low MOIs, but this deficiency can be overcome by infection at high viral titers [145-147]. Experiments in latently-infected mice have also demonstrated that ICP0-deficient viruses have severe reactivation defects, suggesting a role for ICP0 in the establishment of latency and/or reactivation [133, 148-150]. Most intriguingly, several lines of evidence suggest that ICP0 expression is regulated post-transcriptionally by LAT [145, 151-155].

ICP4, another IE protein, is also a transcriptional activator required for the activation of all three classes of HSV-1 gene products [156-158]; viruses deleted for ICP4 do not progress beyond IE gene transcription [157, 159]. ICP4 works synergistically with ICP0 to up-regulate viral transcription and drive HSV-1 through the productive replication cycle [138]. As is the case with ICP0, studies have determined that ICP4 plays a role in reactivation of HSV-1 from latency, and that regulation of ICP4 seems to be exerted at the post-transcriptional level by LAT [145, 152, 160].

### **3.2.5 Latency-associated transcript**

HSV-1 is particularly interesting as a model system for viral latency because, unlike beta- and gamma-herpesviruses, HSV-1 does not produce any viral proteins during latency; the genome is thought to be transcriptionally silent except for the LAT.

Transcription of the LAT is controlled by two latency-associated promoters, LAP1 and LAP2 [161, 162]. LAP1 is a powerful promoter that contains many binding sites for neuronal-specific transcription factors which drives robust LAT expression in

neuronal cells where HSV-1 establishes latency [128, 163]. In contrast, LAP2 is a relatively weak promoter with little neuronal specificity [161]. LAP2 does, however, enable LAT to be expressed at low levels late during productive infection [164]. Very little LAT is expressed early in productive replication due to an ICP4 binding site located within the LAT promoter; *in vivo* and *in vitro* experiments have determined that ICP4 binding down-regulates LAT expression [152, 165-167].

Evaluation of various latently-infected animal systems (e.g., mice, guinea pigs and rabbits) has demonstrated that wild-type HSV-1 establishes latency in significantly more neurons and/or reactivates more efficiently than viruses deleted for the LAT promoter (dLAT); prior to the establishment of latency, there is a brief period of acute replication in infected neurons [168, 169] which ceases soon after infection with wild-type virus [170]. In contrast, dLAT-infected cells continue to produce IE proteins which ultimately results in death of the infected neuron [171]. Congruent to these findings, it was also reported that LAT over-expression prevents virus replication through inhibition of IE gene expression at the mRNA level [145]. These observations fit in neatly with reports that LAT is able to down-regulate expression of both ICP0 and ICP4 [145, 151-155] and suggest that LAT is involved in the establishment of latency and/or virus reactivation [152, 154, 171-176]

The LAT encodes a capped and polyadenylated 8.3 kb mRNA [128, 177] that is spliced to give rise to a 2.0 kb intron and 6.3 kb exonic region [155]. These transcripts are

unusual in that the 2.0 kb intron is highly stable due to a unique lariat structure [178], while the remaining 6.3 kb exonic portion is extremely unstable and rarely detected despite the stabilizing presence of a cap structure and poly(A) tail [155]. As a consequence, the 6.3 kb transcript is present in latent cells at only 5% of the abundance of the 2.0 kb intron [179]. Despite intensive study, it has never been convincingly demonstrated that the 6.3 kb transcript encodes a protein. Therefore, the function this unstable transcript remains unknown.

Several papers have computationally predicted the existence of HSV-1 encoded miRNAs. Pfeiffer *et al.* predicted eight pre-miRNAs in HSV-1, five located within the 6.3 kb LAT [57]. Cui *et al.* predicted the existence of 13 pre-miRNAs potentially encoding up to 24 mature miRNA candidates but were only able to experimentally verify the existence of one, miR-H1, which is located within the LAT promoter [115]. miR-H1, however, is expressed with late gene kinetics and is not produced during latency.

### ***3.3 Materials and methods***

#### **3.3.1 Cell culture and RNA preparation**

293T cells were maintained in DMEM supplemented with 10% FBS. SY5Y cells were maintained in RPMI also supplemented with 10% FBS. Total RNA for 454

sequencing, northern blots, and splint-ligation assays was harvested by TRIzol (Invitrogen).

### 3.3.2 Molecular clones, siRNAs and transfections

pcDNA3/LAT was derived from a EcoRI-BamHI digest of pSG28 which released adjacent EcoRI-BamHI and BamHI-BamHI fragments. Together the fragments contained the entire ~8.3 kb LAT as well as an additional ~130 bp upstream of the TATA box, and ~2.3 kb downstream of the polyadenylation signal. The two fragments were ligated into pcDNA3.1(-)/Zeo (Invitrogen) and carefully screened to verify that the BamHI-BamHI fragment was oriented correctly. Transfection of pcDNA3/LAT into 293T cells was performed using FuGene (Roche).

The ICP0 expression construct (pRS-1) was a very kind gift from Dr. Roz Sandri-Goldin previously described in Sekulovich *et al.* [180]. The ICP0 riboprobe used for northern blot analysis was generated by *in vitro* transcription of a linearized pcDNA3.1(-)/Zeo vector that contained a 431 bp BamHI-XhoI fragment of ICP0 cloned in the reverse orientation. The HA-tagged  $\beta$ -arrestin expression was previously described in Wiegand *et al.* [96].

siRNAs designed to mimic the miR-H2-3p duplex intermediate and the corresponding mutant with 3 mismatches (3M) in the seed region were ordered from IDT along with miR-H6: miR-H2-3p duplex 5' arm: 5'-UCGCACUCGUCCCUGGCUCA GACU-3'; miR-H2-3p duplex 3' arm: CCUGAGCCAGGGACGAGUGCGACU-3';

miR-H2-3p-3M duplex 5' arm: 5'-UCGCACUCGUCCCUGACGCAAACU-3';  
miR-H2-3p-3M duplex 3' arm: 5'-CUUGCGUCAGGGACGAGUGCGACU-3'; miR-H6  
duplex 5' arm: CACUCCCCGUCCUCCAUCCC; miR-H6 duplex 3' arm:  
GAUGGAAGGACGG GAAGUAUA). All plasmid DNA and siRNA co-transfections  
were performed in 293T cells using Lipofectamine 2000 (Invitrogen) according to  
manufacturer directions. Briefly, 293T cells were plated the day before in 24-well plates  
to be 80-90% confluent day of transfection. Cells were co-transfected with either 60-80 ng  
of pRS-1 or 40-60 ng of pSG28 K/B, 30 ng of HA-tagged  $\beta$ -arrestin plasmid, and 10 pmol  
of the appropriate siRNA duplex. One microliter of Lipofectamine 2000 was used per  
well per transfection. Samples were transfected in duplicate and harvested  
simultaneously ~24 hrs post-transfection—one sample was harvested for western blot  
analysis and the other for northern blot.

### **3.3.3 miRNA cloning**

Sample preparation for 454 sequencing was conducted according to the miRNA  
and siRNA cloning protocol as outlined in Lau *et al.* up and including the RT-PCR step  
[17]. After that point, the 454 cloning protocol as outlined by the Hannon lab on the 454  
website was followed ([www.454.com](http://www.454.com)). Initially, 750  $\mu$ g and 60  $\mu$ g of total RNA from  
pcDNA3/LAT transfected 293T cells, and latently infected mouse trigeminal ganglia  
RNA was used, respectively. Sequence data analysis was performed using Microsoft

Excel and a stand-alone BLAST server. cDNAs were identified based on comparisons to both GenBank and miRBase databases.

### **3.3.4 Northern blots and splint-ligation assay**

For ICP0 northern blot, 15 µg of total RNA was run out on a 0.6% agarose gel and transferred onto nitrocellulose. Membranes were fixed by UV irradiation and probed with an ICP0 riboprobe according to standard protocols. Bands were visualized by exposing blots to film at -80°C overnight with intensifying screens. For 2.0 kb LAT intron northern blot, protocol as outline in Alvira *et al.* [181] for an oligonucleotide probe was followed. Splint-ligation assay (USB) was performed with 12 µg of total RNA per sample according to manufacturer directions.

### **3.3.5 Western blots**

Samples were harvested and run out on 10% Tris-HCl gels (Biorad) which were then transferred onto nitrocellulose. Blots were cut in half at the 72 kDa marker band so that the half with the larger proteins could be probed for ICP0 or ICP4 (Virusys) and the half with the smaller proteins could be probed for HA-tagged β-arrestin (Covance). Both halves were then incubated with anti-mouse secondary antibody (GE Healthcare) and bands were visualized with Lumi-Light Western Blotting Substrate (Roche) according to manufacturer directions.

### **3.3.6 Latent HSV infection in mice**

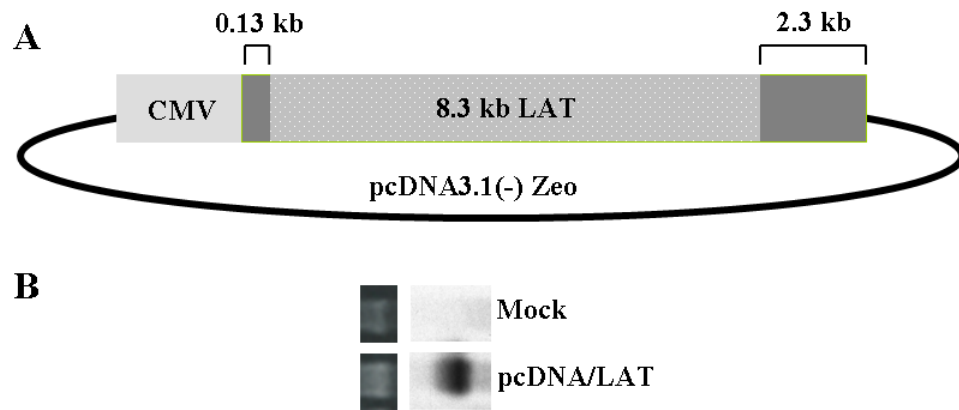
Procedures involving mice were approved by the Harvard Medical School Institutional Animal Care and Use Committee in accordance with federal guidelines. Male CD-1 mice were infected or mock infected, housed for 30 days, and then sacrificed for tissue harvested as previously described.

### **3.3.7 Stem-loop RT-PCR**

Low molecular weight-enriched RNA was isolated with the mirVana miRNA Isolation Kit (Ambion) and the < 40 nucleotide-length fraction isolated using the flashPAGE™ Fractionator System (Ambion). The equivalent of 2.1 µg of total RNA was amplified per reaction. Real time quantitative RT-PCR assays were designed for each miRNA with specific stem-loop RT primers, and PCR primers were purchased from IDT, and TaqMan probes were purchased from Applied Biosystems, Inc (Foster City, CA) (Table A-6). Briefly, RNA was reverse transcribed in duplicate with Multiscribe (Ambion) and the miRNA-specific RT primers. Negative controls included reverse transcriptase-negative, RT primer-negative, and mock-infected experimental samples. Synthetic standard miRNAs were serially diluted and the dynamic range of each assay exceeded 5 orders of magnitude. Aliquots of cDNA were assayed on a PRISM 7700 Sequence Detection System (Applied Biosystems, Inc.). The detection limit was defined by the threshold cycle (Ct) of the negative controls.

### 3.4 Results

Previously successful attempts to clone and identify novel viral miRNAs typically began with small RNAs harvested from virus infected cells that were prepared for cDNA sequencing [57, 58, 182]. The HSV-1 productive replication cycle however, is very efficient (~18 hrs from initial infection to cell death, at high MOIs) and the virus is extremely effective at inhibiting host mRNA and protein synthesis through the degradation of all cellular transcripts [121]. Due the large volume of degradation products contaminating the miRNA pool it would be impossible to clone viral miRNAs from cells infected with wild-type HSV-1. To circumvent this issue, RNA was harvested



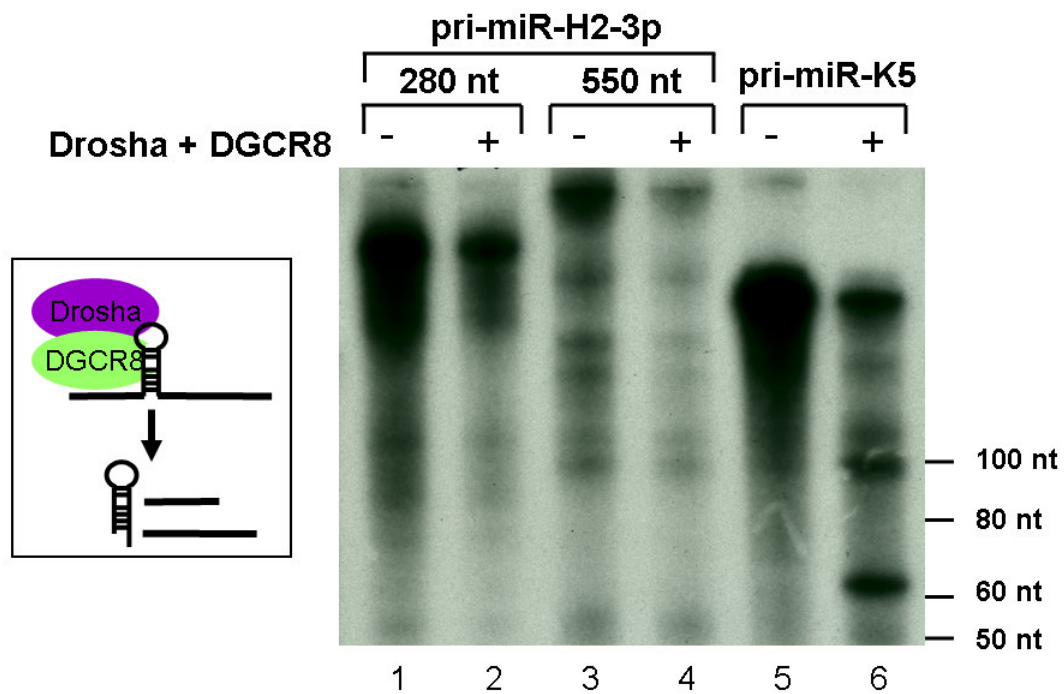
**Figure 3-2. pcDNA/LAT vector.** A) The entire 8.3 kb LAT was cloned into pcDNA3.1(-) Zeo including an extra 0.13 kb upstream of the transcription start site and 2.3 kb downstream of the poly(A) signal. B) Transfection of pcDNA/LAT into 293T cells resulted in ample production of LAT as determined by Northern blot for the 2.0 kb intron. Ethidium bromide staining of rRNA bands serves as a loading control.

from HeLa cells infected with a mutant HSV-1 virus deleted for the *virion host shutoff* (*vhs*) gene—the viral protein responsible for degrading mRNAs [183]. Preliminary sequencing of cDNAs obtained from this sample quickly revealed, however, that in spite of lacking a functional *vhs* gene, this HSV-1 mutant still produced high levels of cellular breakdown products which made screening for novel HSV-1 miRNAs impossible (data not shown).

To avoid this degradation issue and focus on latency-associated miRNAs, a LAT expression vector was constructed. Genomic HSV-1 fragments encompassing the entire 8.3 kb LAT including the native polyadenylation site were excised and ligated into pcDNA3.1(-) to generate pcDNA/LAT (Fig. 3-2A). Northern blot for the 2.0 kb LAT intron verified that the transcript was highly expressed and properly spliced in transfected 293T cells (Fig. 3-2B). Small RNAs from these transfected samples were harvested and used for cDNA cloning. Sequence analysis of ~1500 cDNAs identified nine HSV-1 sequences representing four unique miRNAs of LAT origin, derived from two pre-miRNA hairpins (Table A-1).

It was impossible, however, to verify expression of these putative HSV-1 miRNAs in either pcDNA/LAT transfected cells, or cells infected with wild-type HSV-1; a variety of methods were attempted including northern blot, primer extension, and splint-ligation assays (data not shown). Attempts to generate artificial HSV-1 miRNA expression constructs by a variety of strategies (using both pol II- and pol III-based

vectors) in a variety of cell lines (293T, HeLa, Vero, SY5Y, SK-N-MC) also failed (data not shown). *In vitro* Drosha cleavage assays revealed that recombinant Drosha and DGCR8 were unable to release the putative HSV-1 pre-miRNAs out of either ~280 or ~550 nt *in vitro* transcribed fragments encompassing the precursor hairpins (Fig. 3-3).



**Figure 3-3. Drosha cleavage assay does not release LAT pre-miR-H2-3p hairpin.** *In vitro* transcripts are incubated with recombinant Drosha and DGCR8 which will cleave and release any potential pre-miRNA hairpins present. Cleavage of the KSHV pri-miR-K5 positive control transcript clearly releases a 62 nt hairpin fragment (lane 6). Incubation of Drosha-DGCR8 with either 280 or 550 nt transcripts containing LAT miR-H2-3p, however, does not release the expected 63 nt pre-miRNA (lanes 2 and 4).

In contrast, a ~500 nt positive control fragment containing the KSHV pre-miRNA-K5 hairpin was efficiently cleaved.

These data suggest that the putative HSV-1 miRNAs cloned from pcDNA/LAT-transfected cells may be processed by an alternative mechanism, different than that used to process canonical miRNAs. In an attempt to demonstrate that these HSV-1 miRNAs are legitimate, we performed 454 deep sequencing of small RNAs harvested from latently-infected mouse TGs (Table A-2). This is the first example of miRNA sequencing from a relevant virus-infected tissue *in vivo*.

Deep sequencing returned 254,651 high-quality reads of which 224,729 were 18-24 nts long and occurred at least twice. Of this pool, the vast majority, 204,867 (91.2%), were identified as cellular miRNAs (Table A-3). The most commonly recovered cellular miRNA was miR-34a, a direct transcriptional target of the tumor suppressor protein, p53 [8]; expression of miR-34a induces cell cycle arrest and results in the inhibition of cell proliferation—a defining characteristic of terminally differentiated neurons such as those infected by HSV-1. Other highly expressed neuronal-specific miRNAs found in our TG sample included miR-124, miR-125, miR-138, miR-338 and members of the let-7 family [184].

Of the remaining sequences, 144 (0.1%) were of HSV-1 origin (Table 3-1). These sequences represented three distinct mature miRNAs, each derived from a different pre-miRNA hairpin. Although the final number of HSV-1 miRNAs recovered may seem

**Table 3-1. HSV-1 miRNAs identified through deep sequencing of latently-infected mouse TG.**

Freq	Sequences	Nts	Start	End	Orientation	Designation
20	CCTGAGCCAGGGACGAGTGCGA	22	121871	121892	+	miR-H2-3p
20	GAGCCAGGGACGAGTGCGACTGt	23	121874	121895		
10	CCTGAGCCAGGGACGAGTGCGACT	24	121871	121894		
5	CCTGgGCCAGGGACGAGTGCGA	22	121871	121892		
4	TGAGCCAGGGACGAGTGCGACTGt	24	121873	121895		
3	cGAGCCAGGGACGAGTGCGACTGt	24	121874	121895		
3	GAGCCAGGGACGAGTGCGACTGc	23	121874	121895		
3	CCTGAGCCAGGGACGAGTGCGAC	23	121871	121893		
3	TGAGCCAGGGACGAGTGCGACTG	23	121873	121895		
3	TGAGCCAGGGACGAGTGCGACT	22	121873	121894		
2	CCTGAGCCAGGGACGAGTGCGACa	24	121871	121893		
2	CTGAGCCAGGGACGAGTGCGACT	23	121872	121894		
2	GAGCCAGGGACGAGTGCGACTG	22	121874	121895		
2	TGAGCCAGGGACGAGTGCGA	20	121873	121892		
2	CgGGGACGAGTGCGACTGt	19	121880	121895		
<b>Total</b>	<b>84</b>					
10	CTGGGACTGTGCGGTTGGGA	20	125775	125794	+	miR-H3
2	CaGGGACTGTGCGGTTGGGA	20	125777	125794		
2	CTGGGACTGTGCGGTTGGA	19	125775	125792		
<b>Total</b>	<b>14</b>					
25	CACTTCCCGTCCTTCCATCCC	21	118348	118328	-	miR-H6
11	CACTTCCCGTCCTTCCATCCCa	22	118348	118328		
6	CACTTCCCGTCCTTCCATCC	20	118348	118329		
2	CACTTCCCGTCCTTCCATCCCC	22	118348	118327		
2	CACTTCCCGTCCTTCCATCCct	22	118348	118328		
<b>Total</b>	<b>46</b>					
	<b>144</b>					

low, consider that only ~10% of the cells in the TG are neurons, the specific cell type where HSV-1 establishes latency. Even with an aggressive infection technique (i.e., the use of high titer virus and vigorous corneal scarification), it is only possible to infect approximately 1% to 30% of all neurons. Moreover, miRNA cloning from other herpesvirus-infected cell lines (e.g., EBV and KSHV) has determined that viral miRNAs account for only ~40% of all miRNAs found in infected cells [58]; a higher percentage of viral miRNAs would effectively create a lethal, cellular miRNA-null phenotype. Therefore, the percentage of HSV-1 miRNAs recovered from the TG sample was expectedly low.

Of the three miRNAs cloned from latently infected mouse TGs, only one, miR-H2-3p, had previously been cloned from pcDNA/LAT transfected cells. The other two miRNAs, miR-H3 and miR-H6, were novel. miR-H6 was of particular interest because it is not of LAT origin—it is located antisense to LAT, ~450 bp upstream of the LAT transcription site. As this is beyond the 5' border of the viral sequences contained within pcDNA/LAT (Fig. 3-2A), it was impossible to recover this miRNA from sequencing of the transfected sample.

Deep sequencing was also performed on RNAs harvested from pcDNA/LAT-transfected 293T cells for comparison (Table A-2). Sequencing returned 225,439 high-quality reads of which 185,204 were sequences 18-24 nts long that occurred

**Table 3-2. HSV-1 miRNAs identified through deep sequencing of pcDNA/LAT-transfected 293T cells.**

	Freq	Sequences	Nts	Start	End	Orientation	Designation
	7	TCGCACGC GCCCGGCACAGACT	22	121832	121853	+	miR-H2-5p
	2	TCGCACGC GCCCGGCACAGA	20	121832	121851		
Total	9						
	113	CCTGAGCCAGGGACGAGTGCGA	22	121871	121892	+	miR-H2-3p
	89	CCTGAGCCAGGGACGAGTGCGACT	24	121871	121894		
	27	CCTGAGCCAGGGACGAGTGCGAC	23	121871	121893		
	10	CCTGAGCCAGGGACGAGTGCG	21	121871	121891		
	4	CCTGAGCCAGGGACGAGTGCGACa	24	121871	121893		
	4	TGAGCCAGGGACGAGTGCGACTGa	24	121873	121895		
	3	TGAGCCAGGGACGAGTGCGA	20	121873	121892		
	2	CCTGAGCCAGGGACGAGTGCGAt	23	121871	121892		
	2	GAGCCAGGGACGAGTGCGACTGt	23	121874	121895		
	2	CTGAGCCAGGGACGAGTGCGA	21	121872	121892		
	2	CCTGAGCCAGGGACGAGT	18	121871	121888		
Total	258						
	3	CTGGGACTGTGCGGTTGGGA	20	125775	125794	+	miR-H3
	2	CTGGGACTGTGCGGTTGGGAC	21	125775	125795		
Total	5						
	25	GGTAGAGTTTGACAGGCAAGC	21	125882	125902	+	miR-H4-5p
	11	GGTAGAGTTTGACAGGCAAGCA	22	125882	125903		
	11	GTAGAGTTTGACAGGCAAGCA	21	125883	125903		
	4	GTAGAGTTTGACAGGCAAGC	20	125883	125902		
	2	GGTAGAGTTTGACAGGCAAGCAa	23	125882	125903		
Total	53						
	219	CTTGCCCTGTCTAACTCGCTAGT	22	125927	125948	+	miR-H4-3p
	12	CTTGCCCTGTCTAACTCGCTAG	21	125927	125947		
	9	TTGCCCTGTCTAACTCGCTAGT	21	125928	125948		
	6	CTTGCCCTGTCTAACTCGCTA	20	125927	125946		
	3	CTTGCCCTGTCTAACTCGCT	19	125927	125945		
	2	CTTGCCCTGTCTAACTCGCTAGTa	23	125927	125948		
	2	CTTGCCCTGTCTAACTCGCTAGTt	23	125927	125948		
	2	CTTaGCCCTGTCTAACTCGCTAGT	23	125930	125948		
Total	255						
	29	GTCAGAGATCCAAACCCCTCCGGt	23	126769	126790	+	miR-H5
	5	GTCAGAGATCCAAACCCCTCCGGt t	24	126769	126790		
	5	TCAGAGATCCAAACCCCTCCGGt	22	126770	126790		
Total	39						
TOTAL	619						

at least twice. Of these, 144,955 (78.3%) were cellular miRNAs (Table A-4) and 619 (0.3%) were of HSV-1 origin, representing six mature miRNAs derived from four different pre-miRNA hairpins (Table 3-2).

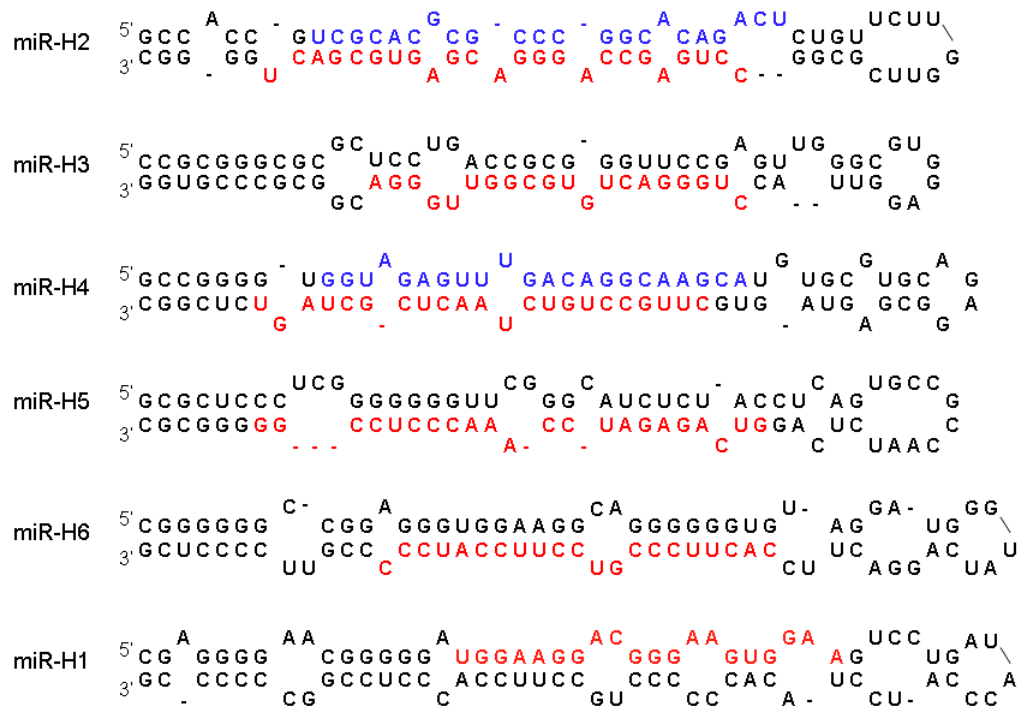
Of these six miRNAs, all except miR-H5 had been previously cloned from either the latently-infected mouse TG sample or pcDNA/LAT-transfected samples. miR-H2-3p and miR-H4-3p were cloned most frequently of all HSV-1 miRNAs recovered.

Secondary structure analysis of putative miRNA sequences demonstrated that all are capable of folding into the characteristic pre-miRNA hairpin structure required for Drosha processing [102] (Fig. 3-4). Where a passenger strand was recovered, the ~2 nt, 3' overhang typical of all duplex intermediates processed by Drosha and Dicer were observed [24].

Expression of all HSV-1 miRNAs was verified using a sensitive stem-loop RT-PCR method [185]. This method differs from traditional RT-PCR in that the primer used for reverse transcription forms a stem-loop at the 5' end while the 3' end remains unstructured and available for binding to the target miRNA. The base stacking of the stem-loop gives the primer better discrimination in differentiating and binding to specific miRNAs. It should be noted, however, that this method requires knowing the exact 3' end of the miRNA being assayed so that the stem-loop primer may be designed accordingly. However, as evident in Table 3-1 and Table 3-2, although the 5' ends of the HSV-1 miRNAs are fairly consistent, there is some variation at the 3' ends. Therefore,

miRNA abundances as determined by this method represent a low estimate of the actual population size.

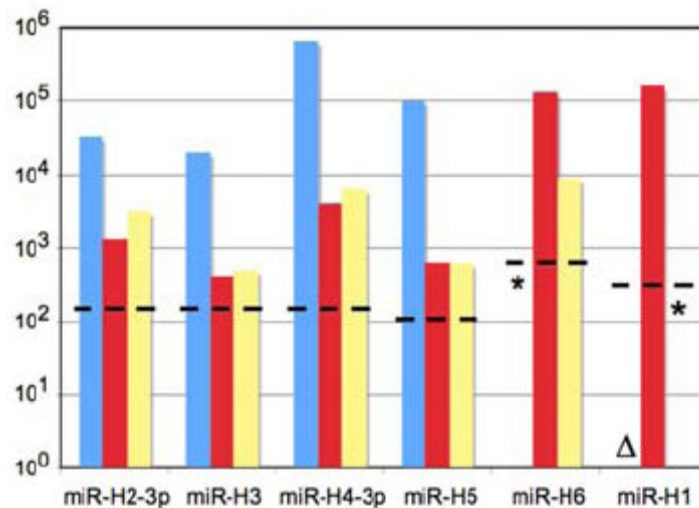
Analysis of Vero cells infected with HSV-1, where the LAT is produced late during productive infection, demonstrated that all HSV-1 miRNAs, including miR-H1, are expressed, albeit at varying levels (Fig. 3-5, red bars). The LAT-derived miRNAs



**Figure 3-4. Secondary structure of HSV-1 miRNAs.** All miRNAs identified by deep sequencing of either latently-infected mouse TG or pcDNA/LAT-transfected 293T cells fold into hairpin structures characteristic of typical pre-miRNAs. miRNA sequences are indicated in red, and, where recovered, passenger strands are indicated in blue.

were detected at  $\sim 10^2$  to  $10^3$  molecules per 2.1  $\mu\text{g}$  of total RNA whereas miR-H1 and miR-H6 were detected at significantly higher levels of  $\sim 10^5$  molecules.

RT-PCR analysis of latently-infected mouse TG samples also confirmed expression of all miRNAs except miR-H1, which, as expected, is not expressed during latency (Fig. 3-5, yellow bars). Interestingly, levels of miRNAs detected during latency ranged from  $\sim 10^2$  to  $10^3$  molecules per 2.1  $\mu\text{g}$  of total RNA regardless of whether or not the miRNAs were LAT-derived—a range of expression far smaller than that observed during productive infection.

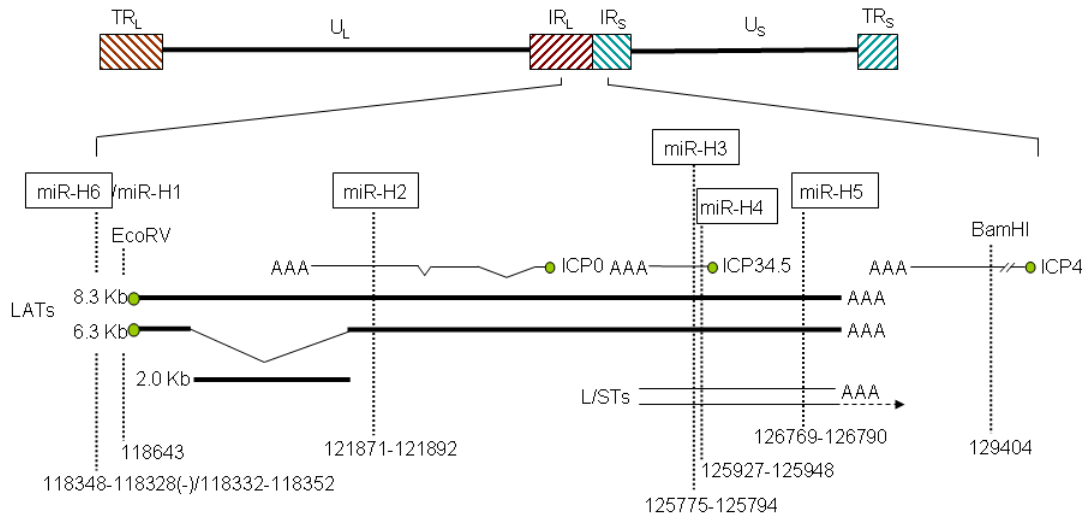


**Figure 3-5. Abundance of HSV-1 miRNAs.** Stem-loop RT-PCR was used to verify the existence and relative abundance of HSV-1 miRNAs from pcDNA/LAT-transfected 293T cells (blue bars), HSV-1-infected Vero cells (red bars) and latently-infected mouse TG (yellow bars). Each sample contained small RNAs derived from 2.1  $\mu\text{g}$  of total RNA. Dashed lines indicate the detection limit as determined for each individual miRNA. ★, not detected;  $\Delta$ , not tested. See Table A-5 for more information.

Genomic mapping of HSV-1 miRNAs revealed that miR-H2-3p is located antisense to *ICP0* and miR-H3 and miR-H4-3p are antisense to *ICP34.5* (Fig. 3-6). This suggests that *ICP0* and *ICP34.5* expression may be regulated by these miRNAs.

Since it was not possible to generate a plasmid-based HSV-1 miRNA expression vectors, we were forced to conduct subsequent experiments using synthetic RNAs mimicking the predicted duplex intermediates. An *ICP0* expression which containing the entire *ICP0* gene including cognate promoter and polyadenylation site was co-transfected with a synthetic miR-H2-3p duplex and an HA-tagged  $\beta$ -arrestin control plasmid. Two samples were transfected in parallel and simultaneously harvested 24 hrs post-transfection.

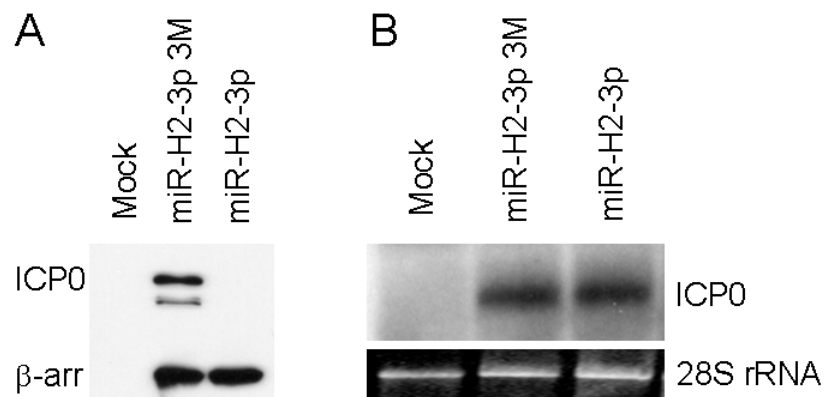
Western blot analysis of one sample demonstrated that miR-H2-3p is capable of potently inhibiting *ICP0* production while a nearly identical siRNA duplex with three mismatches in the seed region, was not (Fig. 3-7A). Northern blot analysis of the parallel sample demonstrated that despite being perfectly complementary to *ICP0*, miR-H2-3p does not appear to cleave and degrade the transcript (Fig. 3-7B). Since potent knock-down of *ICP0* is still observable 48 hrs post-transfection (data not shown) we believe this results from miR-H2-3p acting through a mechanism of translational inhibition, rather than a mechanism of mRNA cleavage that is masked by the rapid transcription of *ICP0* (i.e., *ICP0* mRNAs are cleaved resulting in a drop in protein levels,



**Figure 3-6. Genomic location of HSV-1 miRNAs.** Schematic of the HSV-1 genome is expanded to display details of the LAT locus. Relative sizes, locations and orientations of viral transcripts are as indicated. Sequence coordinates of viral miRNAs and restriction enzyme sites are given according to the HSV-1 strain 17 syn + genome (NC\_001806). Novel miRNAs identified through deep sequencing are boxed. All viral miRNAs are in the same orientation as LAT except miR-H6. pcDNA/LAT contains the entire EcoRV to BamHI fragment. TR, terminal repeat; IR, internal repeat; UL, unique long region; US, unique short region.

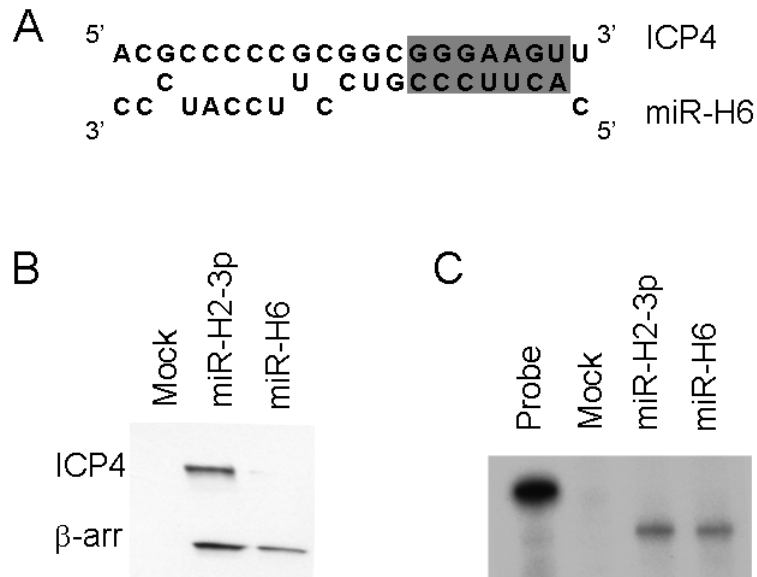
but rapid transcription of *ICP0* driven by the potent viral promoter quickly replenishes the pool of degraded transcripts).

Computational analysis also suggested a high degree of homology between the seed region of miR-H6 and *ICP4* (Fig. 3-8A). To test if miR-H6 affects *ICP4* expression, an *ICP4* expression vector containing the entire *ICP4* gene, native promoter and polyadenylation signal was co-transfected with a synthetic miR-H6 duplex intermediate and an HA-tagged  $\beta$ -arrestin control plasmid. Western blot analysis revealed that



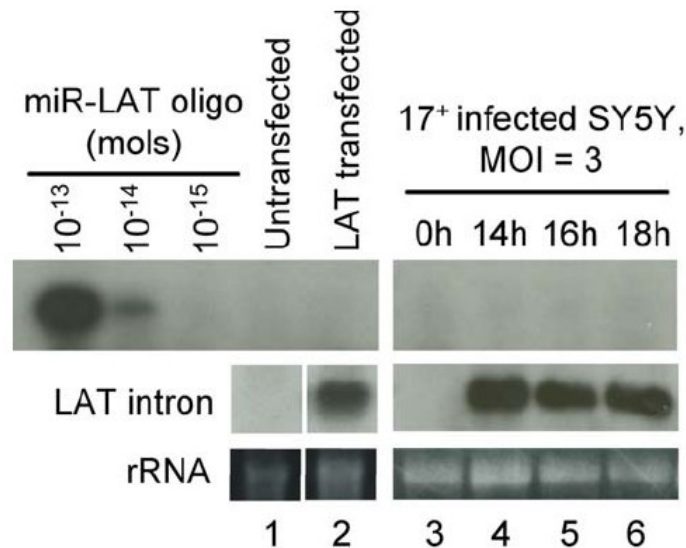
**Figure 3-7. Down-regulation of ICP0 by miR-H2-3p.** 293T cells were co-transfected with *ICP0* and  $\beta$ -arrestin expression plasmids, as well as a synthetic miR-H2-3p duplex or a mutant duplex identical to miR-H2-3p except for three nucleotide mismatches in the seed region (3M). A) Samples were harvested and assayed by western blot for *ICP0* and  $\beta$ -arrestin. B) Samples in panel A were assayed by Northern blot for *ICP0* mRNA levels. Ethidium bromide stained 28S rRNA serves as a loading control.

miR-H6 potently knocks down expression of ICP4 while  $\beta$ -arrestin levels remained unaffected (Fig. 3-8B). (As the seed region of miR-H2-3p does not bind *ICP4*, miR-H2-3p was used as a negative control.) Analysis of *ICP4* mRNA levels by RNase protection assay (RPA) demonstrated that, in spite of the reduction in ICP4 protein levels, mRNA levels remained unchanged (Fig. 3-8C); this result is consistent with a mechanism of miRNA-mediated translational inhibition.



**Figure 3-8. Down-regulation of ICP4 by miR-H6.** A) Sequence homology of miR-H6 to nucleotides 127,298 to 127,318 of *ICP4*. Grey box indicates seed region. B) 293T cells were co-transfected with *ICP4* and  $\beta$ -arrestin expression plasmids, as well as a synthetic miR-H6 duplex or miR-H2-3p negative control. Samples were harvested and assayed by western blot for ICP4 and  $\beta$ -arrestin. C) Samples in panel B assayed by RPA for *ICP4* mRNA levels.

A 2006 paper published by Gupta *et al.* alleged the existence of miR-LAT, an HSV-1 miRNA derived from the LAT transcript [186]. miR-LAT was not identified in our studies or those by Cui *et al.* [115], nor computationally predicted by either Pfeffer *et al.* or Cui *et al.* [57, 115]. All attempts by us and other laboratories to detect miR-LAT failed, including an attempt to precisely reproduce experiments outlined in the original



**Figure 3-9. The purported miR-LAT was not detected in HSV-1-infected cells.** Total RNA was harvested from 293T cells transfected with pcDNA/LAT (lane 2), or SY5Y cells infected with HSV-1 strain 17<sup>+</sup> at an MOI of 3 (lanes 4 to 6). Northern blot analysis for the 2.0 kb intron determined that LAT was highly expressed in both sets of samples. Splint-ligation assay was able to detect a synthetic oligonucleotide identical to miR-LAT at a concentration of 10<sup>-14</sup> mols but was unable to detect miR-LAT in either the transfected or infected samples.

publication where neuronal-like SY5Y cells were infected with HSV-1 strain 17+. Although Northern blot analysis of the 2.0 kb intron indicated robust infection and production of the LAT transcript, miR-LAT was undetectable even though a synthetic oligonucleotide identical to miR-LAT was readily detectable at a concentration as low as  $10^{-14}$  mols (Fig. 3-9). miR-LAT was also undetectable in 293T cells transfected with pcDNA/LAT. A panel of blinded RNA samples provided to the author's laboratory determined that an extremely weak signal detected and presumed to be miR-LAT, was in fact an SY5Y-specific cellular miRNA with limited homology to the miR-LAT sequence; this miRNA was detected in both mock-infected and HSV-1-infected samples. The original publication reporting the existence of miR-LAT has since been retracted [187].

### ***3.5 Discussion***

Since it is encoded within the repeat regions, there are actually two complete copies of the 8.3 kb LAT within the HSV-1 genome [128, 177]. For a 152 kb virus expressing over 80 genes to reserve more than 10% of its genome for one highly conserved gene suggests that LAT plays an important role in the HSV-1 life cycle. For years, study of LAT has focused on the 2.0 kb intron, as it was the only transcript detectable, while the function of the unstable 6.3 kb exonic transcript remained a

mystery. Our findings here provide not only an explanation for the instability of the 6.3 kb LAT, but also attribute a function to the transcript.

In addition to the previously published miR-H1 expressed during productive infection [115], we have now demonstrated that HSV-1 also encodes at least five other miRNAs, four of which are encoded by the LAT. Quantitative RT-PCR determined that these miRNAs are expressed at relatively high levels on a per cell basis during latency, but much lower levels during productive infection (Fig. 3-5).

Genomic mapping of the miRNAs onto the HSV-1 genome revealed that several are located antisense to other viral transcripts (Fig. 3-6), suggesting a role for these miRNAs in regulating viral protein synthesis; miR-H2-3p is antisense to *ICP0* and both miR-H3 and miR-H4-3p are antisense to *ICP34.5*. Through western blot analysis, we demonstrated that a synthetic miR-H2-3p duplex down regulates ICP0 protein levels. Although this result may seem unsurprising, we note that knock-down of target proteins by perfectly complementary siRNAs is not always guaranteed [43]; even well-designed siRNAs fail approximately two-thirds of the time, which is why commercial siRNA libraries actually contain multiple siRNAs against each target [188-191]. Therefore, although we demonstrated inhibition of ICP0 protein production using direct transfection of a synthetic miR-H2-3p duplex, we believe these results are at least suggestive of miR-H2-3p function in a viral context.

Despite potent knock-down of protein levels and a perfect match to the ICP0 transcript, miR-H2-3p does not appear to cleave and degrade the mRNA. As the ICP0 transcript is directly opposite the pre-miR-H2-3p hairpin, it is likely to encode a hairpin structure as well. This *ICP0* hairpin is likely to be a sub-optimal substrate for Drosha and/or Dicer cleavage since no miRNAs were recovered from this strand. However, the secondary structure may be sufficiently complex to prevent miR-H2-3p from fully accessing the site, thereby preventing cleavage; even relatively weak secondary structure around the target region may interfere with siRNA effectiveness [190].

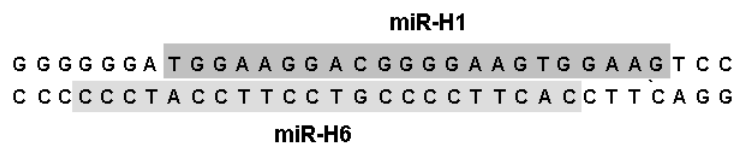
While the minimal seed region required for translational inhibition involves binding by nucleotides 2-8 of the miRNA to the target [34, 35], miR-H6 binds *ICP4* with nucleotides 2-11. Western blot analysis demonstrated that miR-H6 was capable of potently inhibiting production of ICP4 while leaving mRNA levels unchanged, thus demonstrating that miR-H6 also acts through a mechanism of translational inhibition.

What is interesting about the miR-H6 down regulation of ICP4 is the fact that miR-H6 binds *ICP4* in the coding region as opposed to the 3'UTR where miRNAs are believed to generally exert their inhibitory effects [18, 29, 32, 33]. While the binding site is only 67 nts upstream of the *ICP4* stop codon, this would be the first report of an inhibitory miRNA binding in the coding region. We note however, that the majority of research regarding miRNA-induced translational inhibition has focused on binding in the 3'UTR simply because this was true of the first example reported (i.e., *C. elegans*

miRNA *let-7* was found to bind *lin-14* in the 3'UTR [4]), potentially overlooking other miRNAs that bind and exert inhibitory effects from the coding region [1].

The discovery of miR-H6 is exciting for two reasons. Firstly, it demonstrates the existence of another transcript abundantly expressed during latency other than LAT. Although a transcript antisense to LAT has previously been reported, miR-H6 is not contained within the mapped ends of this transcript [192]. Secondly, miR-H6 is directly antisense to the previously reported miR-H1; antisense transcription of miRNAs from a single genetic locus has only recently been reported [193-195]. Of particular interest is the fact that miR-H1 is only expressed during productive infection, while miR-H6 is primarily expressed during latency. This is the first report of bi-directional transcription of a single miRNA locus by a virus.

Although we did not address the issue, it is possible that miR-H3 and miR-H4-3p down-regulate ICP34.5 through an antisense mechanism similar to that observed with



**Figure 3-10. Relative locations of miR-H1 and miR-H6 within the HSV-1 genome.** Schematic demonstrating antisense orientation and overlap of the mature miRNAs. See Fig. 3-6 for genomic coordinates.

miR-H2-3p and ICP0. ICP34.5 is a highly conserved neurovirulence factor that inhibits PKR activation through the dephosphorylation of eIF2 $\alpha$ , preventing the cell-initiated translational arrest that would otherwise hinder virus replication [196-198].

Interestingly, the L/ST transcripts, which are nested within LAT, and are therefore also capable of encoding both miR-H3 and miR-H4-3p, have previously been reported to down-regulate ICP34.5 by a post-transcriptional mechanism [199]. It is not clear what purpose the down regulation of ICP34.5 by either miR-H3 or miR-H4-3p would serve.

We propose a model by which HSV-1 is able to maintain latency by using viral miRNAs to modulate IE protein levels. During latency, miR-H2-3p and miR-H6 repress expression of ICP0 and ICP4, respectively, allowing the virus to remain quiescent within neuronal cells. During reactivation, the ICP0 promoter is activated resulting in an increase in ICP0 protein production. Synergistic interaction between ICP0 and ICP4 increases their levels until sufficient amounts of ICP4 have been produced to bind the LAT promoter and inhibit production of LAT and LAT-derived miRNAs. This releases the virus from latency and allows full productive replication to begin.

In support of our model, we note that LAT is most highly expressed very early during latency [171, 200]. Conversely, the amount of LAT decreases during reactivation from latency [201]. We speculate that these HSV-1 miRNAs need only be expressed at low levels to suppress viral replication. This hypothesis is supported by the fact that HSV-1 seems to be extremely sensitive to ICP0 protein levels; relatively minor changes

in the amount of ICP0 produced can have significant effects on virus reactivation and replication [133, 145-147].

Even though we have now attributed important functions to the unstable 6.3 kb LAT, this does not preclude the 2.0 kb LAT intron from also playing an important role in HSV-1 latency. In fact, due to its highly unusual lariat structure [178] we tend to believe that the intron must have some unique function.

Although we have identified several viral targets for two of the HSV-1 miRNAs, it does not exclude the possibility they, and the other HSV-1 miRNAs, may also target key cellular transcripts. This is particularly true for miR-H5, for which there was no obvious viral target. (Interestingly, it has been determined that miR-H5 is conserved in HSV-2, suggesting an evolutionarily important function for that miRNA.) As it has already been demonstrated that LAT protects neurons from cell death during acute replication prior to latency [172, 202, 203], it will be interesting to determine if any of these miRNAs have a role in the anti-apoptotic pathway. Elucidation of these and other cellular targets should shed more light on the interplay between HSV-1 and host cells during latency.

The discovery of these miRNAs also raises an intriguing possibility in regards to the potential treatment of HSV-1 infected individuals. Current drugs, such as acyclovir, a nucleoside analog which selectively inhibits the replication of herpesviruses, are only able to treat the symptoms of productive infection [204]. Once the virus returns to the

latent state and replication ceases, the drugs are ineffective. Therefore, although we are able to treat people infected with herpesviruses, we are currently unable to cure them. If HSV-1 relies on viral miRNAs to maintain the latent state, then the use of miRNA inhibitors such as antagomirs in infected patients to inhibit miR-H2-3p and miR-H6 could drive HSV-1 out of the latent state and enable permanent elimination of the virus through traditional drug treatment.

## 4. Conclusions and perspectives

It is possible to conceive of three different ways in which a virus may interact with the miRNA/RNAi machinery of a host cell: 1) Viral transcripts/genomes may be cleaved into antiviral siRNAs by the cellular RNAi defense pathway. 2) A cellular miRNA may, by design or coincidence, target viral transcripts/genomes; these cellular miRNAs may either hamper or help the virus. 3) A virus may encode miRNAs that target either viral or cellular transcripts for inhibition.

None of these possibilities are mutually exclusive and the applicable combination is highly dependent on the characteristics of each situation. Here we demonstrate that depending on the virus and cell type infected, the resulting interaction with the host cell miRNA/RNAi system can vary greatly.

### 4.1 Antiviral siRNAs and miRNAs

Viral infection of plants and invertebrates, particularly by RNA viruses, often results in the production of long, perfect dsRNAs that are recognized as foreign and cleaved into antiviral siRNAs. These siRNAs target viral genomes and/or transcripts for degradation and thereby limit virus infection [46, 47]. To counter this cellular defense, many plant and invertebrate viruses encode SRS proteins capable of inhibiting this RNAi response through a wide variety of mechanisms [47, 88-90].

Although the miRNA pathway is highly conserved among plant, invertebrate, and mammalian cells, whether or not mammalian cells retain the ability to use RNAi as an antiviral defense has been a controversial topic [84].

Several recent papers have argued both sides of this issue. Pfeffer *et al.* were the first to examine this question and did not recover any small RNAs of viral origin in cDNA cloning of cells infected with HIV-1, YFV or HCV—suggesting that these RNA viruses neither encode miRNAs nor are processed into siRNAs [57]. Bennasser *et al.* and Omoto *et al.* subsequently published contradictory findings claiming that HIV-1 encodes either an siRNA or miRNA, respectively [85, 86]. Lecellier *et al.* followed with a claim that PFV, another primate retrovirus, is susceptible to inhibition by a cellular miRNA [87]. Our findings are in agreement with those published by Pfeffer *et al.* arguing that primate retroviruses, and more specifically HIV-1, neither encode miRNAs nor generate antiviral siRNAs during infection. We extend these findings by showing that primate retrovirus transactivating proteins HIV-1 Tat, PFV Tas and HTLV-1 Tax do not have SRS properties.

Although it is possible to artificially induce RNAi in mammalian cells [15, 43, 44], it appears that the antiviral RNAi pathway has been supplanted by the more sophisticated innate and adaptive immune responses [52, 53].

An example of a cellular miRNA providing a beneficial effect for a virus, involves miR-122 and HCV; liver-specific cellular miRNA miR-122 binds to the 5'UTR of

the HCV genome and enhances viral replication [205]. Thus far, this is the only known example of such a situation and the mechanism underlying this phenomenon remains unknown.

## **4.2 HSV-1 miRNAs**

Herpesviruses are ideal viral candidates for encoding miRNAs; they are DNA viruses that replicate in the nucleus and establish latent infection that persists for the life of the host [121]. Several herpesviruses have already been shown to encode miRNAs [57-61] and we report here the discovery of five novel HSV-1 miRNAs expressed during latency. Interestingly, only four of these miRNAs are derived from LAT while the fifth is expressed from a novel latency transcript. We have demonstrated that two of these miRNAs, miR-H2-3p and miR-H6, are capable of down-regulating viral proteins ICP0 and ICP4, respectively. As these are IE proteins responsible for activating nearly all other proteins required for productive HSV-1 infection, these miRNAs provide a molecular mechanism for HSV-1 to establish, maintain and transition out of latency.

What makes HSV-1 unique among herpesviruses is that it seems to rely so heavily on viral miRNAs to maintain the latent state. Although the majority of genes encoded by other herpesviruses are also silent during latency, a small subset of proteins and transcripts is produced [114]. HSV-1 in contrast, seems to have the “purest” latency state where no viral proteins and only a few viral transcript are produced. Further elucidation of both viral and cellular targets of these HSV-1 miRNAs will help unravel

the details of latency—the hallmark characteristic of all herpesviruses. Of particular interest will be elucidating the mechanism by which transcription of the HSV-1 latency miRNAs is halted during the transition from latency to productive replication.

## Appendix

**Table A-1. HSV-1 miRNAs identified through standard sequencing of cDNAs isolated from pcDNA/LAT-transfected 293T cells.**

	Freq	Sequences (18-24 nt)	Len	s. start	s. end	Orientation	Designation
	1	TCGCACGCGCCCGGCACAGA	20	121832	121851	+	miR-H2-5p
Total	1						
	4	CCTGAGCCAGGGACGAGTGCGACT	24	121871	121894	+	miR-H2-3p
	1	CCTGAGCCAGGGACGAGTGCGAC	23	121871	121893		
Total	5						
	1	GTAGAGTTTGACAGGCAAGC	20	125883	125902	+	miR-H4-5p
Total	1						
	1	TAGTGCTTGCCGTCTAACTCGT	23	125922	125943	+	miR-H4-3p
	1	CTTGCCGTCTAACTCGCTAGT	22	125927	125948		
Total	2						
TOTAL	9						

**Table A-2. Short RNA sequence analysis.** Overall summary of 454 sequencing data from 293T cells transfected with pcDNA/LAT and latently-infected mouse TG samples.

	<u>293/LAT</u>		<u>TG/HSV-1</u>	
<b>Total reads</b>	225,439		254,651	
<b>Num. of unique sequences</b>	23,683		28,152	
<b>Total num. of sequences 18-24nts</b>	192,864	85.6%	235,114	92.3%
<b>Num. of unique sequences 18-24nts</b>	11,398		15,315	
<b>Total num. of sequences &gt;2 hits</b>	185,204	82.2%	224,729	88.2%
<b>Num. of unique sequences &gt;2 hits</b>	3,738		4,930	
<hr/>				
<b>Total num. of cellular miRNAs</b>	144,955	78.3%	204,867	91.2%
<b>Total num. of non-miRNA sequences</b>	39,603	21.4%	19,862	8.8%
<b>Total num. of HSV-1 miRNAs</b>	619	0.3%	144	0.1%

**Table A-3. Cellular miRNAs identified in latently-infected mouse TG.**

mmu-miR-34a	25463	mmu-miR-181a	187	mmu-miR-324-3p	4	mmu-miR-666-3p	32
mmu-let-7a	2272	mmu-miR-181b	36	mmu-miR-324-5p	127	mmu-miR-666-5p	3
mmu-let-7b	18414	mmu-miR-181c	2	mmu-miR-326	120	mmu-miR-667	46
mmu-let-7c	21409	mmu-miR-182	21	mmu-miR-328	223	mmu-miR-669b	11
mmu-let-7d	325	mmu-miR-183	14	mmu-miR-329	2	mmu-miR-672	16
mmu-let-7e	392	mmu-miR-185	267	mmu-miR-331-3p	34	mmu-miR-674	527
mmu-let-7f	1451	mmu-miR-187	55	mmu-miR-335-5p	2	mmu-miR-676	1151
mmu-let-7g	981	mmu-miR-188-3p	8	mmu-miR-337-5p	41	mmu-miR-700	22
mmu-let-7i	4623	mmu-miR-191	120	mmu-miR-338-3p	10174	mmu-miR-744	425
mmu-miR-1	11	mmu-miR-192	32	mmu-miR-338-5p	24	mmu-miR-760	2
mmu-miR-100	95	mmu-miR-194	98	mmu-miR-341	478	mmu-miR-770-3p	2
mmu-miR-101a	23	mmu-miR-195	500	mmu-miR-342-3p	38	mmu-miR-770-5p	2
mmu-miR-101b	8	mmu-miR-199a-5p	34	mmu-miR-344	5	mmu-miR-7a	28
mmu-miR-103	381	mmu-miR-199b	1381	mmu-miR-345-3p	5	mmu-miR-7b	2
mmu-miR-106a	25	mmu-miR-19b	27	mmu-miR-345-5p	32	mmu-miR-92a	21
mmu-miR-106b	2	mmu-miR-200a	16	mmu-miR-34a	10468	mmu-miR-92b	5
mmu-miR-107	72	mmu-miR-200b	27	mmu-miR-34c	15	mmu-miR-93	573
mmu-miR-124	4053	mmu-miR-200c	8	mmu-miR-350	5	mmu-miR-96	6
mmu-miR-125a-5p	256	mmu-miR-204	3	mmu-miR-351	34	mmu-miR-98	96
mmu-miR-125b-3p	21	mmu-miR-206	3	mmu-miR-370	4	mmu-miR-99a	532
mmu-miR-125b-5p	25332	mmu-miR-20b	29	mmu-miR-376a	4	mmu-miR-99b	30961
mmu-miR-126-3p	763	mmu-miR-21	5724	mmu-miR-378	2	<b>Total</b>	<b>204867</b>
mmu-miR-126-5p	9	mmu-miR-210	1200	mmu-miR-379	13		
mmu-miR-127	3407	mmu-miR-214	301	mmu-miR-383	196		
mmu-miR-130a	252	mmu-miR-217	15	mmu-miR-423-3p	2		
mmu-miR-130b	11	mmu-miR-218	14	mmu-miR-431	84		
mmu-miR-134	7	mmu-miR-221	60	mmu-miR-433	2		
mmu-miR-136	6	mmu-miR-222	37	mmu-miR-434-5p	4		
mmu-miR-138	10140	mmu-miR-23a	17	mmu-miR-449a	11		
mmu-miR-139-3p	18	mmu-miR-23b	32	mmu-miR-451	6		
mmu-miR-139-5p	64	mmu-miR-24	1223	mmu-miR-455	163		
mmu-miR-140	19	mmu-miR-25	161	mmu-miR-466b-5p	2		
mmu-miR-141	2	mmu-miR-26a	2	mmu-miR-466c-5p	6		
mmu-miR-142-3p	136	mmu-miR-26b	2	mmu-miR-466f-5p	9		
mmu-miR-142-5p	10	mmu-miR-27a	433	mmu-miR-466h	23		
mmu-miR-143	760	mmu-miR-27b	412	mmu-miR-484	57		
mmu-miR-144	14	mmu-miR-299	2	mmu-miR-485	2		
mmu-miR-146a	2	mmu-miR-29a	256	mmu-miR-486	275		
mmu-miR-146b	8	mmu-miR-29b	57	mmu-miR-497	1753		
mmu-miR-147	2	mmu-miR-29c	27	mmu-miR-503	29		
mmu-miR-148b	5	mmu-miR-300	111	mmu-miR-511	3		
mmu-miR-149	30	mmu-miR-301a	21	mmu-miR-532-3p	48		
mmu-miR-150	32	mmu-miR-30a	100	mmu-miR-532-5p	24		
mmu-miR-151-3p	4	mmu-miR-30b	458	mmu-miR-540-3p	13		
mmu-miR-151-5p	97	mmu-miR-30c	602	mmu-miR-540-5p	4		
mmu-miR-152	290	mmu-miR-30d	142	mmu-miR-542-5p	3		
mmu-miR-154	5	mmu-miR-30e	33	mmu-miR-551b	63		
mmu-miR-15a	830	mmu-miR-31	22	mmu-miR-574-3p	2656		
mmu-miR-15b	424	mmu-miR-32	4	mmu-miR-574-5p	3		
mmu-miR-16	5680	mmu-miR-322	42	mmu-miR-592	2		
mmu-miR-17	81	mmu-miR-323-3p	9	mmu-miR-665	3		

**Table A-4. Cellular miRNAs identified in pcDNA/LAT-transfected 293T cells.**

hsa-miR-19b	24406	hsa-miR-193b	3	hsa-miR-361-5p	2
hsa-let-7a	570	hsa-miR-194	63	hsa-miR-374a	4
hsa-let-7b	99	hsa-miR-195	48	hsa-miR-374b	12
hsa-let-7c	141	hsa-miR-196a	334	hsa-miR-378	4
hsa-let-7d	16	hsa-miR-196b	144	hsa-miR-421	16
hsa-let-7e	36	hsa-miR-197	63	hsa-miR-423-3p	2
hsa-let-7f	189	hsa-miR-19a	2675	hsa-miR-424	178
hsa-let-7g	109	hsa-miR-19b	4450	hsa-miR-425	25
hsa-let-7i	98	hsa-miR-200c	3	hsa-miR-454	18
hsa-miR-101	8	hsa-miR-20a	3153	hsa-miR-455-3p	27
hsa-miR-103	434	hsa-miR-20b	44	hsa-miR-484	349
hsa-miR-106a	686	hsa-miR-21	599	hsa-miR-486-3p	14
hsa-miR-106b	149	hsa-miR-210	61	hsa-miR-486-5p	129
hsa-miR-107	317	hsa-miR-218	452	hsa-miR-497	17
hsa-miR-10a	731	hsa-miR-221	16068	hsa-miR-503	143
hsa-miR-10b	237	hsa-miR-222	1717	hsa-miR-505	20
hsa-miR-124	21	hsa-miR-224	4	hsa-miR-532-3p	91
hsa-miR-125a-5p	250	hsa-miR-23a	12	hsa-miR-532-5p	38
hsa-miR-125b	216	hsa-miR-23b	21	hsa-miR-542-5p	14
hsa-miR-126	9	hsa-miR-24	51	hsa-miR-548b-3p	62
hsa-miR-127-3p	3	hsa-miR-25	7992	hsa-miR-548d-5p	2
hsa-miR-128b	82	hsa-miR-26a	67	hsa-miR-551a	3
hsa-miR-130b	562	hsa-miR-26b	100	hsa-miR-551b	83
hsa-miR-132	2	hsa-miR-27a	178	hsa-miR-574-3p	72
hsa-miR-133a	2	hsa-miR-27b	477	hsa-miR-589	3
hsa-miR-138	47	hsa-miR-28-3p	16	hsa-miR-598	41
hsa-miR-139-5p	2	hsa-miR-29a	15	hsa-miR-615-3p	34
hsa-miR-140-3p	479	hsa-miR-29b	53	hsa-miR-628-3p	2
hsa-miR-140-5p	2	hsa-miR-29c	16	hsa-miR-660	102
hsa-miR-142-3p	8	hsa-miR-301a	18	hsa-miR-675	15
hsa-miR-146b-5p	2	hsa-miR-301b	10	hsa-miR-7	21
hsa-miR-148b	6	hsa-miR-30a	79	hsa-miR-708	10
hsa-miR-149	10	hsa-miR-30b	1639	hsa-miR-744	69
hsa-miR-151-3p	23	hsa-miR-30c	764	hsa-miR-760	2
hsa-miR-151-5p	58	hsa-miR-30d	95	hsa-miR-767-5p	22
hsa-miR-152	7	hsa-miR-30e	11	hsa-miR-877	2
hsa-miR-15a	713	hsa-miR-31	107	hsa-miR-891a	20
hsa-miR-15b	6007	hsa-miR-32	18	hsa-miR-92a	5766
hsa-miR-16	14523	hsa-miR-320	6	hsa-miR-92b	109
hsa-miR-17	14777	hsa-miR-324-3p	86	hsa-miR-93	9504
hsa-miR-181a	35	hsa-miR-324-5p	34	hsa-miR-935	8
hsa-miR-181b	18	hsa-miR-328	3	hsa-miR-940	2
hsa-miR-181d	2	hsa-miR-330-3p	2	hsa-miR-941	53
hsa-miR-185	76	hsa-miR-331-3p	37	hsa-miR-98	3
hsa-miR-187	79	hsa-miR-338-3p	12	hsa-miR-99a	118
hsa-miR-188-3p	6	hsa-miR-339-5p	22	hsa-miR-99b	10361
hsa-miR-188-5p	2	hsa-miR-340	2	<b>Total</b>	<b>144955</b>
hsa-miR-18a	2069	hsa-miR-342-3p	528		
hsa-miR-18b	37	hsa-miR-346	4		
hsa-miR-191	463	hsa-miR-34a	6049		
hsa-miR-192	119	hsa-miR-361-3p	15		

**Table A-5. Quantitative parameters and representative results of stem-loop RT-PCR of HSV-1 miRNAs.**

miR Assay	miRLAT	miR-H1	miR-H2-3p	miR-H3	miR-H4-3p	miR-H4-5p	miR-H5	miR-H6
Slope (Cycle/log10 molec)	-3.20	-3.16	-3.77	-3.63	-3.66	-3.45	-3.32	-3.43
Intercept (Cycle No.)	45.8	40.8	45.3	42.9	45	42.3	41.7	40.8
Detection limit (molecules/well)	1000	300	150	150	150	50	100	600
HSV (17syn+)/ SY5Y "	<1000	NT	NT	NT	NT	NT	NT	NT
LAT/293T "	NT	NT	32000	20000	630000	200000	100000	<600
HSV (KOS)/Vero "	NT	160000	1300	400	4000	10000	630	130000
HSV (KOS)/Ganglia "	NT	<300	3200	500	6300	<50	630	8600

**Table A-6. Stem-loop RT-PCR reagents for quantification of HSV-1 miRNAs.**

miR-	Reagent	Length	Sequence
all	Reverse Primer	20	CCAGTGCAGGGTCCGAGGTA
miRLAT	RNA Standard	20	UGGCGGCCCCGCCCCGGGGCC
miRLAT	RT-loop	50	GTCGTATCCAGTGCAGGGTCCGAGGTATTGCGACTGGATACGACGGCCCC
miRLAT	For Primer	20	TTATAAATGGCGCCCCGGCC
miRLAT	Probe	18	(6-FAM) CACTGGATACGACGGC (MGB)
H1	RNA Standard	21	UGGAAGGACGGGAAGUGGAAG
H1	RT-loop	50	GTCGTATCCAGTGCAGGGTCCGAGGTATTGCGACTGGATACGACCTTCCA
H1	For Primer	19	CACGCATGGAAGGACGGGA
H1	Probe	18	(6-FAM) TGGATACGACCTTCCACT (MGB)
H2	RNA Standard	24	CCUGAGCCAGGGACGAGUGCGACU
H2	RT-loop	50	GTCGTATCCAGTGCAGGGTCCGAGGTATTGCGACTGGATACGACAGTCGC
H2	For Primer	22	TCATAACCTGAGCCAGGGACGA
H2	Probe	18	(6-FAM) TACGACAGTCGACT (MGB)
H3	RNA Standard	20	CUGGGACUGUGCGGUUGGGA
H3	RT-loop	50	GTCGTATCCAGTGCAGGGTCCGAGGTATTGCGACTGGATACGACTCCCAA
H3	For Primer	18	GCTTGGCTGGGACTGTGC
H3	Probe	18	(6-FAM) TACGACTCCCAACCGCAC (MGB)
H4-3p	RNA Standard	22	CUUGCCUGUCUAAUCGCUAGU
H4-3p	RT-loop	50	GTCGTATCCAGTGCAGGGTCCGAGGTATTGCGACTGGATACGACACTAGC
H4-3p	For Primer	20	GCCTGGCTTGCCTGTCTAAC
H4-3p	Probe	18	(6-FAM) CTGGATACGACACTAGCG (MGB)
H4-5p	RNA Standard	21	GGUAGAGUUUGACAGGCAAGC
H4-5p	RT-loop	50	GTCGTATCCAGTGCAGGGTCCGAGGTATTGCGACTGGATACGACGCTTGC
H4-5p	For Primer	21	GCCCGGGGTAGAGTTTGACAG
H4-5p	Probe	17	(6-FAM) CTGGATACGACGCTTGC (MGB)
H5	RNA Standard	23	GUCAGAGAUCCAAACCCUCCGGU
H5	RT-loop	50	GTCGTATCCAGTGCAGGGTCCGAGGTATTGCGACTGGATACGACACCGGA
H5	For Primer	22	GCCTGGGTCAGAGATCCAAACC
H5	Probe	18	(6-FAM) CTGGATACGACACCGGAG (MGB)
H6	RNA Standard	22	CACUUCCGUCUJCCAUCCCC
H6	RT-loop	50	GTCGTATCCAGTGCAGGGTCCGAGGTATTGCGACTGGATACGACGGGGAT
H6	For Primer	22	TCATAACACTTCCCCTCTTCC
H6	Probe	18	(6-FAM) GGATACGACGGGGATGGA (MGB)

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## Biography

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