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Rapid Communication

Herpes simplex virus 1 microRNAs expressed abundantly during latent infection are not essential for latency in mouse trigeminal ganglia

Martha F. Kramer [a], Igor Jurak [a], Jean M. Pesola [a], Sandrine Boissel [a,1], David M. Knipe [b], Donald M. Coen [a,*]

[a] Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA
[b] Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115, USA

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ABSTRACT

Several herpes simplex virus 1 microRNAs are encoded within or near the *latency associated transcript* (*LAT*) locus, and are expressed abundantly during latency. Some of these microRNAs can repress the expression of important viral proteins and are hypothesized to play important roles in establishing and/or maintaining latent infections. We found that in lytically infected cells and in acutely infected mouse ganglia, expression of *LAT*-encoded microRNAs was weak and unaffected by a deletion that includes the *LAT* promoter. In mouse ganglia latently infected with wild type virus, the microRNAs accumulated to high levels, but deletions of the *LAT* promoter markedly reduced expression of *LAT*-encoded microRNAs and also miR-H6, which is encoded upstream of LAT and can repress expression of ICP4. Because these *LAT* deletion mutants establish and maintain latent infections, these microRNAs are not essential for latency, at least in mouse trigeminal ganglia, but may help promote it.

Introduction

Herpes simplex virus (HSV) infections of mammalian hosts have two distinct phases, productive ("lytic") infection – which results in abundant expression of >70 genes, viral replication, cell death, and virus spread – and latent infection (reviewed in Roizman et al., 2007). Following inoculation at a peripheral site, the virus lytically infects epithelial cells, from which it gains access to nerve terminals. After retrograde transport to neuronal cell bodies, the virus can initiate a second round of lytic infection (acute ganglionic replication), and/or establish a latent infection. Latent infection persists for the lifetime of the host, and is the reservoir for recurrent HSV disease.

Lytic gene expression begins with the expression of immediate early (α or IE) genes, whose transcription does not require the expression of any viral proteins. IE proteins such as ICP4 and ICP0 are important for the expression of subsequent viral genes. IE gene expression is followed by the expression of early (β or E) genes, which are required for viral DNA replication, and then the expression of late (γ or L) genes, whose maximal transcription requires viral DNA synthesis (Honess and Roizman, 1974). In contrast, latent infections are characterized by the absence of infectious virus and little detectable expression of IE, E, and L genes. The only abundantly expressed locus encodes the latency-associated transcripts (LATs).

In latently infected neurons, LATs include a low abundance primary 8.3 kb polyadenylated transcript and highly abundant, stable ~2 kb and smaller intron species (Dobson et al., 1989; Farrell et al., 1991; Krause et al., 1991; Spivack and Fraser, 1988) (Fig. 1E). During lytic infection, only the ~2 kb LAT intron species is detected, it is much less abundant than in latently infected cells, and its maximal expression requires viral DNA synthesis (Krause et al., 1988; Spivack and Fraser, 1987; Spivack and Fraser, 1988).

Several results point to products of the *LAT* locus functioning in repression of lytic HSV gene expression, which would favor establishment and maintenance of latency. There is evidence that LAT can repress lytic replication and IE gene expression in a neuronal cell line (Mador et al., 1998). *LAT* deletion mutations increase lytic HSV gene expression in acutely and latently infected mouse trigeminal ganglia (Chen et al., 1997; Garber et al., 1997) (although there is a report to the contrary in rabbit ganglia (Giordani et al., 2008)). Although there is a report of the opposite effect (Kwiatkowski et al., 2009), we have found that LAT deletions also result in decreased heterochromatin marks on lytic gene promoters in latently infected mouse trigeminal ganglia, so LAT has been proposed to silence viral gene expression as a long non-coding RNA (Cliffe et al., 2009; Wang et al., 2005).

A second potential mechanism for repression of lytic gene expression during latency was uncovered with the discovery of HSV-encoded microRNAs (miRNAs) in ganglia latently infected with either HSV-1 or HSV-2 (Jurak et al., 2010; Tang et al., 2008, 2009; Umbach et al., 2008, 2009, 2010). MiRNAs are ~22 nucleotide (nt) RNAs derived from larger (~60–80 nt) hairpin precursors known as pre-miRNAs, which are in turn derived from long primary transcripts (pri-miRNAs) (reviewed in Bartel, 2004). MiRNAs specifically recognize

* Corresponding author. Fax: +1 617 432 3833.
E-mail address: Don_Coen@hms.harvard.edu (D.M. Coen).
[1] Present address: Department of Biochemistry, University of Washington, Seattle, WA 98195, USA.

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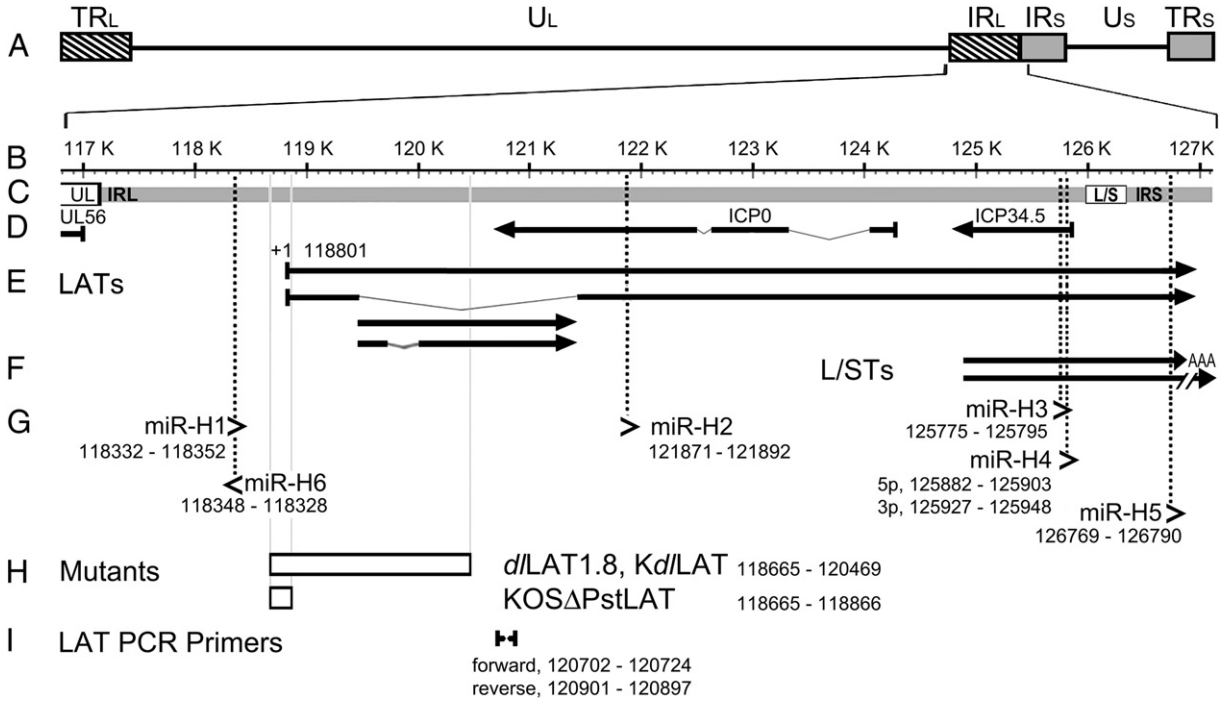

Fig. 1. Genetic elements in the HSV-1 long internal repeat. (A) Prototypic arrangement of the HSV-1 genome with the terminal and internal repeat regions (TR_L, IR_L, striped boxes; IR_S, TR_S, solid boxes) bracketing long and short unique (U_L, U_S, lines) regions. (B) IR_L region expanded with genomic nt number in kb (K). (C) U_L (open box)/IR_L (gray band) junction, IR_L/IR_S junction (L/S, open box), and start of IR_S (gray band). (D) Protein coding transcripts from this region that encode U_L56 (only 5′ end of transcript is shown), ICP0, and ICP34.5. (E) LATs, including unstable 8.3 kb and 6.3 kb minor LATs and 2 kb and 1.5 stable LAT intron species. (F) L/ST RNA species. (G) Sites of miR-H1 through miR-H6 indicated with vertical dotted lines and their directions indicated by open arrows. (H) Mutants used in this study, with deletions indicated by open boxes. (I) Sites of LAT PCR primers used in this study to quantify LAT RNA. In (D), (E), and (F), transcripts are depicted with black lines with arrowheads indicating direction of transcription. For protein coding transcripts and stable LATs, gray lines indicate sequences that are spliced out. In (E), (G), (H), and (I), locations are indicated in nucleotide number.

target mRNAs and inhibit their translation and/or promote their degradation (Bartel, 2009). In transient transfection assays, one of the HSV miRNAs found in latently infected ganglia, miR-H2 (for HSV-2, also known as miR-III), can repress the expression of the important IE protein ICP0 (Tang et al., 2009; Umbach et al., 2008), and a second miRNA, HSV-1 miR-H6, can repress the expression of the essential IE protein ICP4 (Umbach et al., 2008). Two others, miR-H3 and miR-H4 (for HSV-2, also known as miR-I and miR-II) can repress the expression of the L protein ICP34.5 in transient transfection assays (Tang et al., 2008, 2009, I. Jurak, S. Lim, P.J. Yen, and D.M. Coen, unpublished data). Four of the miRNAs found in latently infected ganglia (miR-H2–miR-H5) could derive from the long, lower abundance LATs (Fig. 1), and were detected in cells transfected with LAT expression plasmids (Umbach et al., 2008; Tang et al., 2008, 2009). However, a fifth miRNA found in latently infected ganglia, HSV-1 miR-H6, was not detected in cells transfected with the *LAT* transcription unit. This miRNA is encoded upstream of the *LAT* promoter, on the opposite strand from *LAT* transcribed sequences (Umbach et al., 2008) (Fig. 1G). The pri-miRNA for HSV-1 miR-H6, and the sequences that control its expression have not been identified.

Because HSV-1 miR-H2–H4 and miR-H6 can repress the expression of proteins that are crucial for lytic gene expression and virus production, we and others have hypothesized that these miRNAs play important roles in establishing and/or maintaining latency (Moens, 2009; Tang et al., 2008; Umbach et al., 2008). On the other hand, most *LAT* deletion mutants are able to establish and maintain latent infections (Krause et al., 1995; Leib et al., 1989a; Perng et al., 1994; Sedarati et al., 1989; Steiner et al., 1989; Thompson and Sawtell, 1997). We wondered whether *LAT* deletions actually affect the expression of HSV-1 miRNAs, particularly miR-H6.

We therefore investigated the expression of HSV-1 miRNAs encoded within or near the *LAT* locus in cell culture and in acutely and latently infected mouse ganglia. In particular, we wished to determine how the expression of these miRNAs relates to HSV-1 LAT expression, including whether LAT is the pri-miRNA in these settings for these miRNAs, as proposed, and whether *LAT* mutations affect the expression of miR-H6, which is not in the *LAT* transcription unit. Our results have important implications for the role(s) of miRNAs in latency.

Results and discussion

Kinetics of HSV-1 miRNA expression in cell culture

We wished to evaluate the kinetics of expression of HSV-1 miR-H2–H6 in lytically infected cells, and the relationship of the expression of these miRNAs to LAT expression. We also assayed a sixth HSV-1 miRNA, miR-H1, which is encoded near the *LAT* locus (Cui et al., 2006; Jurak et al., 2010). Using Northern blotting, we had previously shown that in lytically infected cells HSV-1 miR-H1 and miR-H6 are abundantly expressed, that the pre-miRNA for HSV-1 miR-H5 can be detected, and that miR-H1 behaves as a late gene product (Cui et al., 2006; Jurak et al., 2010). The expression of HSV-1 miRNAs in lytically infected cells has also been assessed using reverse transcription-polymerase chain reaction (RT-PCR), but either at only one time point, 16 h.p.i., in which numbers of miRNAs per cell were measured (Umbach et al., 2008), or at one or two time points (e.g. 6 and 18 h.p.i.), in which relative increases in miRNA were measured (Umbach et al., 2009).

As a first step, we performed Northern blot hybridization of short-enriched (S) RNA (<200 nt) from Vero cells infected with HSV-1 wild type (wt) strain KOS for times ranging from two to 18 h.p.i. on two separate blots (Figs. 2A and B). Use of S RNA allowed us to run the equivalent of 70 to 100 μg total RNA per lane, to increase the sensitivity of detection. Both blots were sequentially probed, stripped and re-probed. The cellular miRNA, *let-7a*, served as both a cellular and a loading control (bottom panels, Figs. 2A,B), and showed no change

in abundance during infection, as observed previously (Cui et al., 2006). For all the HSV-1 miRNAs shown in Figs. 2A and B, and for miR-H5 (Fig. 2D; Cui et al., 2006; Jurak et al., 2010 and data not shown), ~60 nt hybridizing RNA species, which we infer are pre-miRNAs, could be detected. All but pre-miR-H3 were not detected until at least 8 h.p.i., consistent with late kinetics (Figs. 2A and B, and data not shown). As previously observed (Cui et al., 2006; Jurak et al., 2010), mature miRNAs were detected for miR-H1 and miR-H6 (Figs. 2A, B). We also detected reproducibly, albeit very weakly, mature miR-H2 at 18 h.p.i. (Fig. 2A, second panel from top). (Although we appeared to detect miR-H2 at 4 h.p.i. in Fig. 2A, this result could not be reproduced.) Similar to previous results with HSV miRNAs (Cui et al., 2006; Jurak et al., 2010; Tang et al., 2008, 2009), in all cases the mature miRNAs were substantially less abundant than the pre-miRNAs (Figs. 2A and B). As discussed previously (Cui et al., 2006), this might be due to HSV interfering with miRNA biogenesis or stability, which would be consistent with HSV-1 suppression of RNA-induced gene silencing (Wu et al., 2009). Alternatively, HSV pre-miRNAs might be poor substrates for Dicer, or HSV miRNAs might be relatively unstable.

In the presence of the protein synthesis inhibitor cycloheximide (Chx) or the viral DNA synthesis inhibitor acyclovir (ACV), pre-miR-H1, -H2, -H4-5p, and -H6 and mature miR-H1 (as previously reported (Cui et al., 2006)), -H2, and -H6 were not detectable (Figs. 2A and B). Thus, these RNAs behave as late gene products by both time course and inhibitor criteria.

In contrast to the other HSV-1 pre-miRNAs, pre-miR-H3 was evident at 2 h.p.i. Its abundance remained relatively constant through 8 h.p.i., and then increased between 8 and 18 h.p.i. (Figs. 2A, B). At no time was mature miR-H3 detectable. Interestingly, the abundance of pre-miR-H3 was enhanced at 8 h.p.i. in the presence of Chx. Thus, by both time course and inhibitor criteria, this pre-miRNA behaves as an immediate early gene product. Perhaps this pre-miRNA is expressed from an IE promoter called αX that is negatively regulated by ICP4 and drives the expression of transcripts known as L/STs (Bohenzky et al., 1993, 1995; Yeh and Schaffer, 1993; Fig. 1). Pre-miR-H4 and pre-miR-H5 could also be derived from L/STs, yet we did not detect IE kinetics for these pre-miRNAs. Differential primary RNA secondary structure, differential utilization of the miRNA biogenesis machinery, or differential effects on degradation of the pri-miRNA following excision of pre-miR-H3 may help explain this observation. More study of the biogenesis of these pre-miRNAs is needed.

Consistent with the increase in abundance between 8 and 18 h.p.i., ACV reduced the abundance of pre-miR-H3. This result suggests that pre-miR-H3 accumulates through late times, as is true for certain other IE RNAs such as *ICP0* mRNA (Weinheimer and McKnight, 1987).

HSV-1 miRNAs are expressed from a LAT mutant during lytic infection

HSV-1 miR-H2 through miR-H5 are encoded within the 8 kb *LAT* transcription unit and are expressed in cells transfected with plasmids encoding full-length LAT (Umbach et al., 2008) (Fig. 1E). To investigate whether these miRNAs are derived from LATs during lytic infection, Vero cells were infected with wt strain KOS, a KOS-derived deletion mutant, K*dl*LAT, which lacks the LAT promoter and ~1.8 kbp of LAT coding sequences, and a marker-rescued derivative of K*dl*LAT, KFSLAT (Fig. 1H; Garber et al., 1997). At 16 h.p.i., S RNA was isolated and analyzed by Northern blot hybridization (Figs. 2C and D). Roughly equal amounts of mature *let-7a* were detected in each sample (Figs. 2C, D, bottom panels). Moreover, similar amounts of pre-miRNAs and mature miRNAs for miR-H1, miR-H2 and miR-H6 and pre-miRNAs for

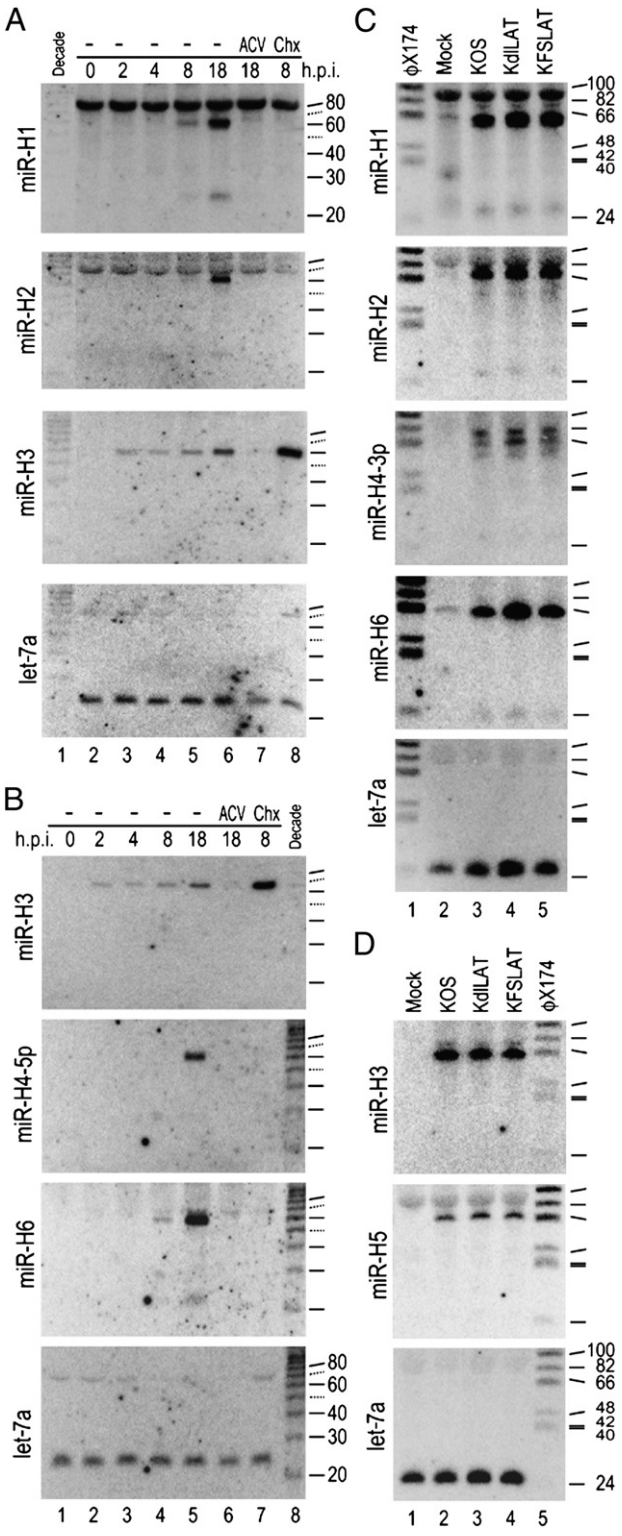

Fig. 2. Northern blot analysis of HSV-1 pre-miRNAs and mature miRNAs in lytically infected cells. S RNAs harvested from mock- or HSV-infected cells were separated by polyacrylamide gel electrophoresis, transferred to nylon membranes, and probed with miRNA-specific end-labeled ^{32}P oligonucleotides, as indicated to the left of each panel. Each blot was stripped, exposed to film to verify that stripping was complete, and re-probed multiple times. Different intensities of end-labeled ^{32}P molecular weight markers indicate the relative order of probing. The sizes of molecular weight markers are indicated with tick marks to the right of each panel. (A and B) Time course of infection. Mock-infected (0) or HSV-1 infected cell RNA at 2, 4, 8, and 18 h.p.i.; ACV, cells infected in the presence of acyclovir and harvested at 18 h.p.i.; Chx, cells infected in the presence of cycloheximide and harvested at 8 h.p.i. Decade, Decade Marker (Ambion). (C and D) Effect of *LAT* deletion. Cells were mock-infected (Mock) or infected with wt HSV-1 strain KOS, LAT deletion mutant K*dl*LAT1.8, or its rescued derivative, KFSLAT, and harvested at 16 h.p.i. ϕX174, ϕX174 HinfI molecular weight marker.

miR-H3, miR-H4-3p and miR-H5 were detected in cells infected with wt KOS, K*dl*LAT, and KFSLAT, with any minor differences among the samples correlating with similar differences in the amounts of *let-7a*, indicative of differences in loading. Thus, accumulation of these pre-miRNAs and miRNAs during lytic infection is independent of the *LAT* promoter.

Quantitative RT-PCR analysis of miRNA expression during lytic infection

To analyze miRNA expression during lytic infection more quantitatively, we performed stem–loop quantitative RT-PCR (RT-qPCR) analysis of RNA from Vero cells infected with HSV-1 strain KOS for times ranging from two to 18 h.p.i. in the presence or absence of ACV. Because pre-miRNAs are much more abundant than mature miRNAs (Fig. 2), <40 nt RNA species were isolated to prevent pre-miRNAs and larger transcripts from contributing to the RT-qPCR measurements. (Such larger species are detected by this technique, albeit less efficiently (Chen et al., 2005; M.F.K., unpublished results)). We assayed both strands (5p and 3p) generated from pre-miR-H4, because they are similar in abundance. Cellular *let-7a* miRNA was quantified to normalize recovery. Background values for the stem–loop RT-qPCR assays ranged from ~0.1–~10 molecules per cell, depending on the miRNA assayed (shown as zero time points in Fig. 3). We caution that stem–loop RT-qPCR assays may underestimate the abundance of certain miRNAs, especially if there is heterogeneity in miRNA 5′ and 3′ ends. Results from three independent experiments were pooled (Fig. 3).

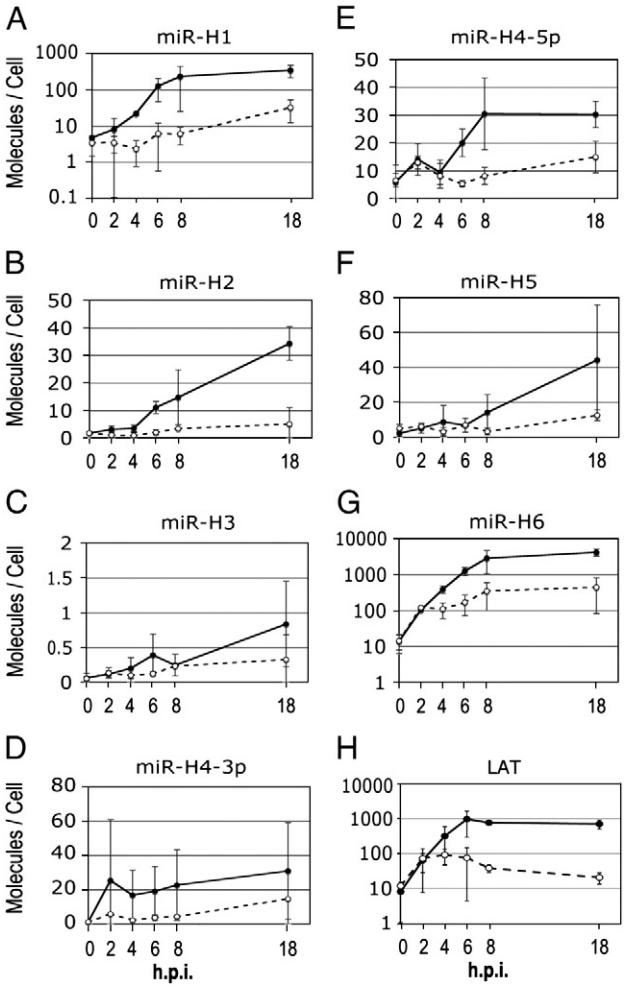

Fig. 3. RT-PCR analysis of miRNAs and LAT in HSV-1 infected Vero cells. Vero cells were infected with wt HSV-1 without (filled circles, solid line) or with (open circles, dashed line) 200 μM ACV. MiRNA values were normalized to *let-7a*, and LAT values were normalized to ng total RNA, and are presented as numeric means ± standard deviation (n = 3) of molecules per cell plotted against h.p.i. (A) miR-H1; (B) miR-H2; (C) miR-H3; (D) miR-H4-3p; (E) miR-H4-5p; (F) miR-H5; (G) miR-H6; (H) LAT.

Using this more sensitive technique, some of the miRNAs, including miR-H1, mir-H4-3p, and miR-H6, could be detected as early as 2 or 4 h.p.i. Consistent with our Northern blot analyses and our previous results (Fig. 2 and Umbach et al., 2008), miR-H1 and miR-H6 accumulated throughout infection and were substantially more abundant than the other HSV-1 miRNAs (Figs. 3A and G). In this analysis, we found ~300 and ~4000 molecules/cell of miR-H1 and miR-H6, respectively, which differ from our previously reported values of ~1200 and ~300 molecules/cell, respectively (Umbach et al., 2008). These differences likely stem from changes to our assays (Supplemental materials and methods) and experimental variability. ACV reduced the expression of miR-H1 and miR-H6 ~10-fold. Thus, based on the Northern blot and qRT-PCR results, the unknown pri-miRNA for miR-H6 during productive infection behaves as a late transcript.

miR-H2, miR-H4-3p, miR-H4-5p, and miR-H5, accumulated to ~30 molecules/cell, and miR-H3 to less than 1 molecule/cell. That might be interpreted to mean that these miRNAs would be less likely to exert meaningful effects on their targets. However, expression of both strands of miR-H4 was relatively high at 2 and 4 h.p.i., at which times the abundance of the L *ICP34.5* mRNA is relatively low. The expression of miR-H2–miR-H5 exhibited varying degrees of sensitivity to ACV, ranging from ~10-fold for miR-H2 to ~2-fold (if any) for miR-H3 (although this apparent lack of sensitivity to ACV may merely reflect difficulties in quantifying this poorly expressed miRNA.) Thus, the RT-qPCR and Northern blot analyses identify all of the miRNAs except pre- and mature miR-H3 as late gene products.

To compare the expression of the miRNAs to that of LAT, total RNA from which the <40 nt RNA had been isolated was assayed for LAT intron species. LAT accumulated to much higher levels (~1000 molecules/cell) than any of the miRNAs encoded within the LAT transcription unit, and its expression exhibited more sensitivity to ACV (about 30-fold) than did any of the miRNAs.

Thus, based on differential ACV sensitivity (Fig. 3), and, more importantly on the lack of effect of the *LAT* deletion (Fig. 2), transcripts other than LATs serve as pri-miRNAs for HSV-1 miR-H2–miR-H5 during lytic infection, even though these miRNAs are encoded within the *LAT* transcription unit. The pri-miRNAs appear to be late transcripts, except for pri-miR-H3. During HSV infection, many long transcripts appear to arise from inefficient polyadenylation/cleavage (e.g. Holland et al., 1984). Any number of such transcripts that originate upstream of LATs, including those from promoters of transcripts described by Singh and Wagner (1993) and Zhu et al. (1999) that we have suggested might serve as pri-miRNAs for miR-H1 (Cui et al., 2006), might also serve as pri-miRNAs for miR-H2–miR-H5 during productive infection.

Low expression of miRNAs during acute infection of mouse ganglia

We next investigated the expression of HSV-1 miR-H1–miR-H6 during acute ganglionic infection in two independent experiments. Mice were inoculated on the cornea with wt strain KOS, *LAT* mutant virus *dl*LAT1.8, which contains the same 1.8 kb deletion as K*dl*LAT, and its marker rescued derivative, FSLAT (Leib et al., 1989a) (Fig. 1H). At three days p.i., at which time titers of infectious virus are maximal (Leib et al., 1989b), HSV-1 DNA was measured using qPCR and normalized to mouse DNA. HSV-1 miRNAs were quantified from <40 nt RNAs with stem–loop RT-qPCR and normalized to cellular *let7a* (4.4×10^9 copies/ganglion). Although amounts of viral DNA were different between experiments, within each experiment amounts of wt and mutant DNA were similar (Supplemental Table 1).

The levels of HSV-1 miRNAs in ganglia acutely infected with KOS were more than 3 orders of magnitude lower than those of *let7a*, with no more than 3×10^6 copies/ganglion on average (data from one of the two experiments are provided in Table 1; similar results were

Table 1
Levels of HSV-1 miRNAs in acutely and latently infected mouse trigeminal ganglia.

	miR-H1	miR-H2	miR-H3	miR-H4-3p	miR-H4-5p	miR-H5	miR-H6
Acute	5.5 ± 0.3	6.5 ± 0.4	*	5.3 ± 0.4	5.0 ± 0.2	5.4 ± 0.05	6.3 ± 0.1
Latent	*	7.8 ± 0.1	*	8.7 ± 0.1	7.4 ± 0.5	8.5 ± 0.09	8.0 ± 0.5
L/A	N/A	19	N/A	2400	260	1300	52

Levels of HSV-1 miRNAs in trigeminal ganglia of mice infected with wt strain KOS at 3 (acute, n = 2) or 30 (latent, n = 3) days after infection, expressed as $\log_{10}$ mean molecules per ganglion ± standard deviation. L/A, ratio of mean levels of miRNA in latently infected ganglia to those in acutely infected ganglia (numeric). The differences in levels of miR-H2, -H4-3p, -H4-5p, -H5, and -H6 between acutely infected and latently infected ganglia were statistically significant ($p \leq 0.0202$, two-sided *t* test).
*Not detectable above background. N/A, does not apply.

obtained in the second experiment). We could not detect one of these miRNAs, miR-H3, above background levels. The miRNA levels were also very low on a per viral DNA basis; no miRNA was present in ganglia acutely infected with KOS at >2 copies/viral genome (Fig. 4). There were no significant differences between the levels of the miRNAs per viral genome in ganglia acutely infected with KOS and those in ganglia infected with *dl*LAT1.8 (Fig. 4). Thus, averaged over the entire ganglion, at the peak of ganglionic replication during acute infection of mice, expression of these miRNAs was weak, and as was true during lytic infection in cell culture, independent of the *LAT* promoter.

However, some infected cells in acutely infected ganglia do not undergo lytic infection, but rather enter the latent program of gene expression and thus contain only low levels of HSV DNA (Simmons et al., 1992). In these cells, the amounts of miRNAs are likely to be relatively high per viral genome. This might be even more likely after day 3 p.i. (see below), and thus the miRNAs would be much more likely to exert effects on target mRNAs. In particular, miRNAs targeting *ICP4* and *ICP0* mRNA should result in reductions in the subsequent expression of IE and early proteins, favoring entry into latency. Indeed, miRNA levels in acutely infected cells might set a threshold: when IE mRNAs are expressed below this threshold, latency would be more likely to be established; when IE mRNAs rise above this threshold, productive infection would be more likely to ensue.

Expression of HSV-1 miRNAs during latent infection

We next investigated the expression of HSV-1 miR-H1–miR-H6 using stem–loop RT-qPCR in trigeminal ganglia infected with wt strain KOS at 30 days p.i. We were unable to detect miR-H3 above background values in these studies (data not shown). We note that miR-H3 has been detected much more abundantly in deep sequencing analyses of latently infected mouse and human ganglia than in productively infected cells (Jurak et al., 2010; Umbach et al., 2008, 2009). Thus, we consider our inability here to detect miR-H3 above background in latently infected ganglia to be a shortcoming of the RT-qPCR assay. We were also unable to detect HSV-1 miR-H1 above background values, but its levels were at least ten-fold lower than those of the other miRNAs (data not shown). We therefore focused on those miRNAs: HSV-1 miR-H2, miR-H4-5p, miR-H4-3p, miR-H5, and miR-H6.

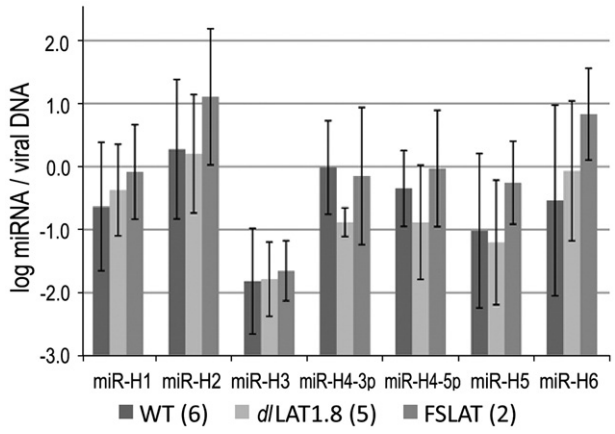

Fig. 4. A *LAT* deletion does not affect expression of HSV-1 miRNAs in acutely infected mouse ganglia: In two independent experiments, mice were infected with wt HSV-1 strain KOS (darkest bars), mutant *dl*LAT1.8 (lightest bars), or rescued derivative, FSLAT (intermediate bars). At 3 days p.i., ganglia were harvested and analyzed for each miRNA indicated below the graph, and for viral DNA. The amounts of each miRNA per viral DNA are presented. Each bar represents the $\log_{10}$ mean; error bars indicate standard deviations. No significant differences were observed between the mutant and either the wt or rescued derivative using one way ANOVA with Bonferroni post-tests applied to each miRNA.

In one experiment, we compared levels of these miRNAs to amounts of viral DNA ($\log_{10}$ mean of 4.8 ± 0.5 molecules/ganglion), using qPCR, and amounts of stable LAT, using qRT-PCR ($\log_{10}$ mean of 7.7 ± 0.2 molecules/ganglion). In this experiment, there were roughly 20 to 200 of these miRNAs per viral genome in latently infected ganglia (Table 2). Moreover, expression was relatively consistent from ganglion to ganglion (results not shown), in contrast to lytic transcripts that have been detected in latently infected ganglia (Chen et al., 1997, 2002; Feldman et al., 2002; Kramer and Coen, 1995; Pesola et al., 2005). These miRNAs were expressed at levels ranging from 2% to 20% those of stable LAT (Table 2). The reduced amounts of miRNAs relative to LATs could reflect inefficient processing of the miRNAs from the pri- and pre-miRNAs, or reduced stability of the miRNAs relative to stable LAT. Regardless, the abundance and consistent expression of these miRNAs during latency indicate that they are truly latent transcripts — and perhaps should be considered LATs.

In one of the two experiments in which we had examined miRNA expression in acutely infected ganglia, we compared the amounts of HSV-1 miRNAs in acutely infected and latently infected ganglia (Table 1). In this experiment, there was substantially (19- to 2400-fold) more of miR-H2 and miR-H4–miR-H6 per ganglion in latently infected ganglia than in acutely infected ganglia, and these differences were statistically significant (Table 1). This substantial increase in miRNA levels between 3 days p.i. and 30 days p.i. differs from the similar levels of LAT at the two time points (Kramer et al., 1998). This difference may reflect relatively slow processing of the miRNAs from their pri- and pre-miRNAs. It also suggests that the levels of miRNAs and thus the probability that they exert repressive effects increase after 3 days p.i.

Effects of LAT mutations on miRNA expression during latent infection

We next addressed the effect of the 1.8 kbp *LAT* deletion in mutant *dl*LAT1.8 on expression of the HSV-1 miRNAs in latently infected ganglia. We inoculated mice via the corneal route with *dl*LAT1.8, its rescued derivative, FSLAT, or wt strain KOS, and analyzed ganglia 30 days p.i. for DNA by qPCR and for miRNAs by stem–loop RT-qPCR. Three independent experiments were performed, and the results were pooled (Supplemental Table 1 and Fig. 5A). Similar to our previous findings using older PCR methods (Chen et al., 1997, 2000, 2002) and with recent studies of *LAT* mutants in mice that used real-time methods (Hoshino et al., 2009), we found similar amounts of DNA (~3×10^4 molecules/ganglion) in ganglia latently infected with wild type, mutant, and marker rescued viruses (Supplemental Table

Table 2
Levels of HSV-1 miRNAs relative to LAT and viral DNA in latently infected mouse trigeminal ganglia.

	miR-H2	miR-H4-3p	miR-H4-5p	miR-H5	miR-H6
miRNA/vDNA	170	130	19	19	54
miRNA/LAT	0.20	0.14	0.022	0.021	0.060

Mean numbers of miRNAs per viral genome (vDNA) or LAT in trigeminal ganglia from mice infected with HSV-1 wt strain KOS (n = 4).

1). This result differs from one from a study using different virus and mouse strains where less viral DNA was found in *LAT* mutant-infected ganglia, but does not preclude there being fewer latently infected neurons in *LAT* mutant-infected ganglia as found in that study (Thompson and Sawtell, 2001). The amounts per viral genome of miR-H2, miR-H4-5p, miR-H4-3p, miR-H5, and miR-H6 were markedly reduced in ganglia latently infected with the *LAT* mutation, *dl*LAT1.8, relative to ganglia infected with either the wt or the rescued derivative (Fig. 5A). The reductions ranged from 500- to 900-fold, and were statistically significant, except for the difference in miR-H4-5p between *dl*LAT1.8 and KOS (Fig. 5A). Indeed, the levels of these miRNAs in *dl*LAT1.8-infected ganglia were not distinguishable from background (one way ANOVA with Bonferroni post-tests). Similar results were obtained using a different mutant, K*dl*LAT, containing the 1.8 kbp *LAT* deletion (Garber et al., 1997), and its marker rescued derivative (not shown). Thus, the 1.8 kbp *LAT* deletion essentially abolishes the expression of these miRNAs.

The dramatic reductions of miR-H2 and miR-H4–miR-H6 in *dl*LAT1.8-infected ganglia could have been due to the loss of sequences downstream of the *LAT* promoter (Fig. 1). To address this, we conducted an experiment comparing the expression of these miRNAs from wt strain KOS, mutant KOSΔPstLAT, and its marker-rescued derivative PstRLAT. KOSΔPstLAT contains a ~200 bp deletion that includes the LAT promoter, leaving almost the entire 8 kb LAT transcribed sequence intact (Cliffe et al., 2009; Fig. 1H). This deletion, like the 1.8 kb deletion of *dl*LAT1.8, reduces LAT expression by at least three orders of magnitude (Chen et al., 1997; Cliffe et al., 2009). Our results with KOSΔPstLAT (Fig. 5B) were very similar to those with *dl*LAT1.8 (Fig. 5A). In ganglia latently infected with KOSΔPstLAT, there were marked (>100-fold) and significant reductions in the levels of all of these miRNAs relative to the wt virus and the rescued derivative (Fig. 5B). None of the amounts of HSV-1 miRNAs in ganglia latently infected with KOSΔPstLAT was significantly higher than background (one way ANOVA with Bonferroni post-tests).

Thus, the 200 bp sequence that contains the *LAT* promoter is necessary for the expression of miR-H2 and miR-H4–miR-H6. This result combined with the result that HSV-1 miR-H2 through miR-H5 can be expressed from a plasmid with the *LAT* transcription unit driven by a heterologous promoter (Umbach et al., 2008) provides strong evidence that LAT is the pri-miRNA for these miRNAs. LAT is also highly likely, then, to be the pri-miRNA for HSV-1 miR-H7 and miR-H8, which are encoded within the *LAT* transcription unit and have been detected in latently infected mouse and human ganglia (Jurak et al., 2010; Umbach et al., 2009, 2010).

More surprising was our finding that a 200 bp deletion that includes the LAT promoter drastically reduces expression of miR-H6 (Fig. 5B). The simplest interpretation of this result is that a crucial segment of the promoter for the miR-H6 pri-miRNA lies within those 200 bp. An intriguing possibility is that this promoter and the LAT promoter are identical. Recently, many eukaryotic promoters have been shown to mediate bidirectional transcription (reviewed in Beretta and Morillon, 2009; Jacquier, 2009). On the other hand, it is possible that the promoter for pri-miR-H6 is within *LAT* transcribed sequences, but the *LAT* promoter deletion somehow reduces pri-miR-H6 expression. Consistent with this possibility, the 5′ end of a transcript that could serve as the pri-miRNA for miR-H6 maps within the LAT 5′ exon, outside of the sequences deleted in KOSΔPstLAT (D.C. Bloom, personal communication). Other interpretations such as LAT expression being required for miR-H6 expression are less likely, but cannot currently be excluded.

Similarities and differences with HSV-2

It is interesting to compare our results on HSV-1 miRNAs with similar studies of HSV-2 miRNAs. Our conclusion that HSV-1 miR-H2–miR-H5 in lytically infected cells derive mainly from a pri-miRNA other than LAT is similar to the conclusion reached by Tang et al. (2008, 2009) for HSV-2 miR-H2–miR-H4 (also known as miR-III, I, and II, respectively). In a paper that appeared while this manuscript was being completed, it was reported that HSV-2 miR-H6 expression in cell culture was unaffected by a 0.6 kbp deletion of *LAT* promoter and first exon sequences (Tang

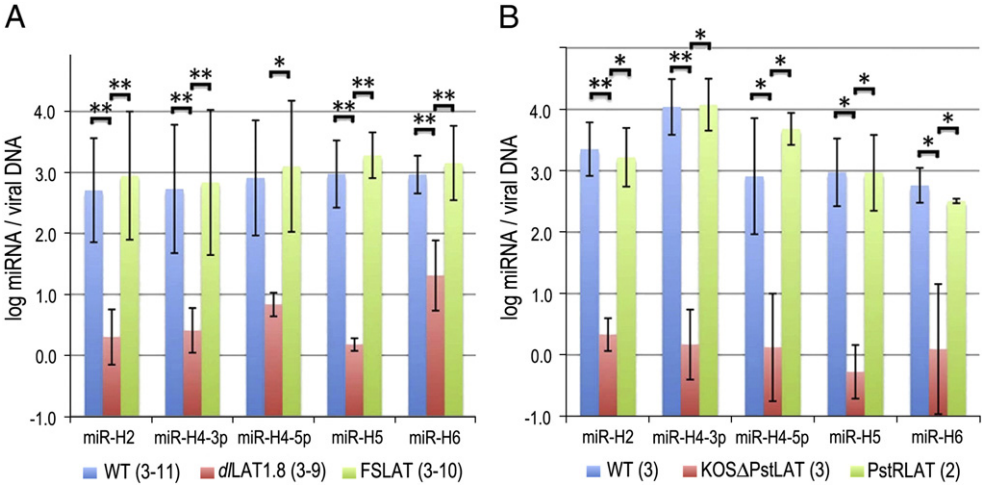

Fig. 5. *LAT* deletions markedly reduce the expression of HSV-1 miRNAs in latently infected mouse ganglia. In three independent experiments, mice were infected with wt HSV-1 strain KOS (blue bars), mutant *dl*LAT1.8 (red bars in (A)) or PstΔLAT (red bars in (B)), or rescued derivatives, FSLAT (green bars in (A)) or PstRLAT (green bars in (B)). At 30 days p.i., ganglia were harvested and analyzed for each miRNA indicated below the graphs, and for viral DNA. The numbers of ganglia infected with each virus that were analyzed are provided in parentheses under the graph. The amounts of each miRNA per viral DNA are presented. Each bar represents the $\log_{10}$ mean; error bars indicate standard deviations. Highly significant differences (**, $p<0.001$) and significant differences (*, $p<0.05$) between mutant and wt or mutant rescued derivatives are indicated as determined by one way ANOVA with Bonferroni post-tests applied to each miRNA.

et al., 2011), similar to our findings with a larger *LAT* deletion. Our finding that a 200 bp deletion of the *LAT* promoter or a larger *LAT* deletion drastically impairs expression of HSV-1 miR-H2, and miR-H4–miR-H6 in latently infected cells is similar to the effect of the larger 0.6 kbp deletion of *LAT* promoter and first exon sequences on HSV-2 miR-H3 (miR-I) (Tang et al., 2008), and HSV-2 miR-H6 (Tang et al., 2011). However, in contrast to our finding that *LAT* deletions had little effect on expression of HSV-1 miRNAs in acutely infected mouse ganglia, Tang et al. reported that the expression of HSV-2 miR-H3 and miR-H6 was drastically reduced by the 0.6 kbp deletion in acutely infected guinea pig ganglia (Tang et al., 2008, 2011). Moreover, inspection of data in these studies (Tang et al., 2008) reveals 2- to 3-fold higher amounts of HSV-2 miR-H3 (miR-I) than LAT in guinea pig ganglia latently infected with HSV-2, and similar amounts of HSV-2 miR-H3 and miR-H6 in acutely and latently infected ganglia. In contrast, we found that the HSV-1 miRNAs were at least five-fold less abundant than LAT in mouse ganglia latently infected with HSV-1, and we found much lower levels of the miRNAs in acutely infected ganglia. Some of the differences among the studies might be due to technical issues. These issues could include when acute ganglionic infection was assayed (day 3 for mice and day 8 for guinea pigs), that HSV-2 miRNA levels were measured from total RNA using an assay that may have also detected pri-miRNAs and pre-miRNAs, or the possibility that our stem–loop RT-qPCR assays somehow considerably underestimated miRNA abundance. Alternatively, some of the discrepancies among the studies might reflect interesting differences between HSV-1 and HSV-2 and/or between mouse and guinea pig.

Implications for HSV latency

Because HSV-1 miR-H6 is encoded upstream of *LAT* and is able to repress ICP4 expression in transient transfection assays (Umbach et al., 2008), we wondered whether HSV *LAT* deletion mutants such as *dl*LAT1.8 might express this miRNA, thus explaining why these mutants are able to establish and maintain latent infections, at least qualitatively (Krause et al., 1995; Leib et al., 1989a; Perng et al., 1994; Sedarati et al., 1989; Steiner et al., 1989; Thompson and Sawtell, 1997). However, we found that such mutants are also highly defective for miR-H6 expression, as well as for miRNAs encoded within *LAT* transcribed sequences. Thus, the miRNAs expressed abundantly during latency are not necessary for establishment and maintenance of latent infections, at least in mice. On the other hand, the defect of HSV-1 *LAT* mutants for miRNA expression can help explain why such mutants exhibit reduced repression of productive cycle genes during acute and latent ganglionic infection (Chen et al., 1997, 2002; Garber et al., 1997). Additionally, *LAT* mutants are defective for chromatin-mediated transcriptional silencing (Cliffe et al., 2009; Wang et al., 2005) (although one study differed (Kwiatkowski et al., 2009)), which may be a second repressive LAT function. Why then are these mutants able to establish and maintain latent infections in mice? One possibility is that other factors such as innate immunity (e.g. Leib et al., 1999), the repressive effects of immune cells in ganglia (e.g. Knickelbein et al., 2008), or the neuronal environment (e.g. Kristie et al., 1999) are sufficient for establishment and maintenance of latency. Another possibility is that while certain of the miRNAs affected by the *LAT* mutations might be necessary for repression of lytic gene expression and thus latency, others might exert opposite effects, perhaps keeping the latent virus poised for reactivation. Studies of mutations that affect individual miRNAs should help address this possibility. A final consideration is that in humans, unlike in mice, spontaneous reactivation of HSV is frequent (Margolis et al., 2007; Mark et al., 2008; Schiffer et al., 2009). It is possible that interference with *LAT* functions, including miRNAs (e.g. by oligonucleotides that antagonize miRNAs), could have a greater effect in humans, and thus disrupt latency.

Materials and methods

Viruses and cells

Viruses used in this study were HSV-1 strain KOS and mutants derived from KOS: *dl*LAT1.8 and its marker-rescued derivative FSLAT (Leib et al., 1989a), K*dl*LAT and its marker-rescued derivative KFSLAT (Garber et al., 1997) and KOSΔPstLAT and its marker-rescued derivative PstRLAT (Cliffe et al., 2009). Viruses were propagated and titrated on Vero cells, as previously described (Leib et al., 1989a).

Northern blot analysis

Vero cells were mock-infected or infected with HSV-1 strain KOS, K*dl*LAT, or KFSLAT at a multiplicity of infection (MOI) of 10. At various times post infection, cells were harvested, and S RNA (≤200 nt) was isolated using the mirVana kit (Ambion) per the manufacturer's instructions. In preliminary experiments, we quantified total RNA, isolated with the same kit, and S RNA spectrophotometrically (NanoDrop) and determined that S RNA represented 10% and 14% of total RNA in trigeminal ganglia and Vero cells, respectively (data not shown). Up to 10 μg of Vero cell S RNA was resolved by polyacrylamide gel electrophoresis on a 15% gel, transferred to a positively-charged nylon membrane (BrightStar-Plus, Ambion), and UV-cross-linked using a Stratagene UV Crosslinker on the auto crosslinking setting (120,000 μJ/cm^2). DNA oligonucleotides complementary to each miRNA of interest were obtained from IDT (sequences provided in Supplemental Table 2) and end-labeled with [γ-^{32}P]-ATP. Hybridization with the labeled oligonucleotides, washes, and radiographic detection were performed as described previously (Cui et al., 2006).

Animal procedures and tissue acquisition

Male CD-1 mice obtained from Charles River Laboratories were housed in accordance with institutional and NIH guidelines on the care and use of animals in research, with procedures approved by the Institutional Animal Care and Use Committee of Harvard Medical School. HSV (2×10^6 PFU in 3 μL per eye) or cell culture media was inoculated onto scarified corneas of 7 week old mice, as described previously (Kramer and Coen, 1995). At three (acute) or thirty (latent) days post infection, mice were sacrificed humanely, and the trigeminal ganglia were rapidly harvested onto dry ice or into liquid N_2, and stored at −80 °C as described previously (Kramer and Coen, 1995).

qPCR and qRT-PCR

In describing our real-time PCR experiments, we conform to guidelines that have been designated minimum information for publication of quantitative real-time PCR experiments (MIQE; Bustin et al., 2009). How nucleic acids were prepared and assessed for quality, our choices for reference gene, assay design and validation, reagents and instruments, and data analysis for stem–loop RT-qPCR are detailed in Supplemental materials and methods. Briefly, viral (*tk* gene) and reference (cellular single-copy *adipsin* gene) DNA were quantified by qPCR as described previously (Pesola et al., 2005), but with mouse *adipsin* rather than *GAPDH* as the cellular gene (sequences provided in Supplemental Table 3). LAT was quantified by RT-qPCR from total RNA samples using RT-qPCR procedures described previously for other genes (Kramer et al., 2003) (sequences provided in Supplemental Table 3). LAT was normalized to ng total RNA, and copies per cell were calculated from experimentally determined total RNA content. Total RNA content in Vero cells, which we experimentally determined to be 19.2 pg total RNA/cell, was ascertained by extraction of total RNA from known numbers of Vero cells counted using a hemocytometer, and quantified spectrophotometrically (NanoDrop). Stem–loop RT-qPCR

assays (Chen et al., 2005) to quantify host and viral miRNAs were extensively modified from those originally described (Umbach et al., 2008) to optimize detection and quantification and reduce background. These modifications are detailed in Supplemental materials and methods. Briefly, <40 nt RNA was assayed for viral miRNAs and for *let7-a*, which served as a normalization control.

Supplementary materials related to this article can be found online at doi:10.1016/j.virol.2011.06.027.

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