Patterns of accumulation of miRNAs encoded by herpes simplex virus during productive infection, latency, and on reactivation

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The key events in herpes simplex virus (HSV) infections are (*i*) replication at a portal of entry into the body modeled by infection of cultured cells; (*ii*) establishment of a latent state characterized by a sole latency-associated transcript and microRNAs (miRNAs) modeled in murine peripheral ganglia 30 d after inoculation; and (*iii*) reactivation from the latent state modeled by excision and incubation of ganglia in medium containing anti-NGF antibody for a timespan of a single viral replicative cycle. In this report, we examine the pattern of synthesis and accumulation of 18 HSV-1 miRNAs in the three models. We report the following: (*i*) H2-3P, H3-3P, H4-3P, H5-3P, H6-3P, and H7-5P accumulated in ganglia harboring latent virus. All but H4-3P were readily detected in productively infected cells, and most likely they originate from three transcriptional units. (*ii*) H8-5P, H15, H17, H18, H26, and H27 accumulated during reactivation. Of this group, only H26 and H27 could be detected in productively infected cells. (*iii*) Of the 18 we have examined, only 10 miRNAs were found to accumulate above background levels in productively infected cells. The disparity in the accumulation of miRNAs in cell culture and during reactivation may reflect differences in the patterns of regulation of viral gene expression during productive infection and during reactivation from the latent state.

regulation of gene expression | alpha genes | beta/gamma genes | transcription

A key property of herpes simplex viruses (HSVs) is that they are able to persist in infected individuals for life, notwithstanding formidable opposition by innate and adaptive immunity of the host. In brief, HSV is transmitted by physical contact between tissues of an uninfected individual with tissues of an infected individual containing active lesions. From the site of infection, usually oral or genital mucosa, the virus is transported retrograde to sensory or autonomic neurons in which it establishes a latent infection. In some individuals, the virus replicates and is transported retrograde to a site at or near the site of initial infection (reviewed in ref. 1). Early studies have shown that during the latent state, HSV expresses two overlapping noncoding RNAs, 2.0 and 1.5 Kb in size (2, 3). These RNAs, designated latency-associated transcripts (LATs), have been linked to stability of the latent virus (4, 5). Recently, Umbach et al. discovered by deep sequencing that HSV encodes a set of seven microRNAs (miRNAs) and that most of these are present in latently infected neurons (6). In subsequent studies, the number of potential virus-coded miRNAs in HSV-1– and HSV-2–infected cells increased to 27 (miRBase; www.mirbase.org/cgi-bin/mirna_summary.pl?org=hsv1). Some were shown to be present both in productively infected cells and in murine ganglia harboring latent virus (refs. 7–12 and reviewed in ref. 13).

The discovery of HSV miRNAs raised many questions regarding their function and the requirements for their synthesis both in the course of productive infection and during the latent state. Recent developments in our laboratory have enabled us to address some of these questions. Specifically, we have shown that infected cells export exosomes along with infectious virus particles. The exosomes were found to contain mRNA as well as miRNAs (14). The experimental design of this study involved measuring the accumulation of miRNAs unimpeded by any metabolic inhibitors, in the presence of cycloheximide or actinomycin D. RNAs accumulating in the presence of actinomycin D validated the hypothesis that they were introduced by exosomes during the infection and not made after infection (14). miRNAs that accumulated above the amounts introduced into cells in the presence of cycloheximide are those that do not require viral protein synthesis to be made. They are in effect similar to α or immediate early viral gene products. The miRNAs made only or predominantly in the absence of inhibitors resemble the β (early) or γ (late) gene products—i.e., products that require α proteins to be made. Finally, we identified a small number of miRNAs that were made in larger amounts in the absence of de novo protein synthesis. These miRNAs were designated as pre-α miRNAs, indicating that their synthesis in productive infection is blocked by one or more α gene products.

The second development relevant to these studies is the availability of a murine model for analyses of the events leading to virus reactivation (15, 16). In principle, reactivation takes place in two steps. In the first step, the viral genome residing in neurons is derepressed and expresses its genes. In the second step, the newly formed infectious virions are transported anterograde to the portal of entry or spread to satellite cells (1). The focus of this study was the miRNAs that accumulate during the first step that is within a time frame of a single replicative cycle—i.e., 18–24 h after initiation of the reactivation process. In our model, mice are infected by the corneal route. After 30 d, the trigeminal ganglia (TG) are excised and either analyzed immediately

Significance

The mechanism by which HSV enters into a silent, latent state in neurons remains a major problem in virology. The only manifestations of viral gene expression during latency are the accumulation of a noncoding transcript and a set of microRNAs (miRNAs). Available data support the hypothesis that these play a role in the maintenance of the latent state and predict the accumulation of a different set of miRNAs during latency and reactivation. This report (*i*) supports the hypothesis that miRNAs arise during latency from multiple transcriptional events on the basis of location and differences in transcriptional regulation in productively infected cells and (*ii*) specifically identifies miRNAs that arise during reactivation and that differ from those accumulating in productively infected cells.

Author contributions: T.D., Z.H., G.Z., and B.R. designed research; T.D. performed research; Z.H. contributed new reagents/analytic tools; T.D., Z.H., G.Z., and B.R. analyzed data; and G.Z. and B.R. wrote the paper.

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or incubated in medium containing anti-NGF antibody to synchronize the reactivation process. In this model, viral mRNAs representative of α, β, and γ groups begin to accumulate at ~5 h after excision and incubation of the ganglia (15). Analyses of miRNAs accumulating in ganglia immediately after excision, and after 24 h of incubation led us to identify miRNAs accumulating during latency and those that accumulated predominantly on reactivation. By default, we also identified miRNAs that could not be detected in ganglia during the latent state or reactivation.

The preceding literature predicted 27 HSV miRNAs. The experimental studies reported to date focused on miRNAs accumulating in infected cells, in ganglia after initial inoculation, and in ganglia harboring latent virus (refs. 6–12; miRBase, www.mirbase.org/cgi-bin/mirna_summary.pl?org=hsv1).

The data vary considerably. Umbach et al. reported six miRNAs in ganglia harboring latent virus (H2-3P, H3, H4-3P, H4-5P, H5, and H6 in ganglia) and the same subset plus H1 in productively infected cells (6). Other studies reported as many as 15 miRNAs in lytically infected cells and 10 miRNAs in ganglia harboring latent virus (7–12). None reported the temporal pattern and requirements for accumulation of miRNAs in productively infected cells or in the course of reactivation of latent virus in murine ganglia.

Here we report the results of studies on 18 miRNAs encoded by HSV-1(F). We report that the patterns and requirements for the accumulation of miRNAs in productively infected cells differ significantly. We also report that, although the pattern of accumulation of miRNAs in ganglia harboring latent virus are with minor exceptions similar to that first reported by Umbach et al. (6), a totally different set of miRNAs accumulates in the course of HSV reactivation from latent state.

Results

Accumulation of miRNAs in Productively Infected Cells. As noted in the Introduction, exosomes generated in infected HEp-2 cells carry RNA in addition to other macromolecules from infected to newly infected cells. The delivered payload is a very useful baseline on which to measure the accumulation of newly synthesized miRNAs in infected cells as a function of time after infection.

In the series of experiments described here, replicate Vero cell cultures were exposed to 10 pfu of purified virus per cell, prepared as described elsewhere, and incubated in medium containing mixture 199 supplemented with 1% calf serum (14). Duplicate cultures were incubated in medium containing either cycloheximide (100 μg/mL) or actinomycin D (10 μg/mL). Replicate cultures of cells incubated in medium without inhibitors or in the presence of cycloheximide were harvested at 0 (mock infection), 1, 3, 6, or 12 h after exposure to the virus. Actinomycin D served to inhibit de novo synthesis of RNAs and therefore measured the amounts delivered to the infected cells by exosomes. Replicate cultures incubated in medium containing actinomycin D were harvested at 0, 3, and 6 h after exposure to the virus. The procedures for extraction and quantification of viral miRNAs are described in *Materials and Methods*.

To simplify the presentation of the results, the patterns of accumulation of individual miRNAs have been placed in four groups and are presented in Figs. 1 and 2. They were as follows: The amounts of miRNAs accumulating in the presence of actinomycin D (Figs. 1 and 2) reflect the minimal amounts brought into the infected cells via exosomes in the virion preparations during infection. The data show that, although some miRNAs accumulating in the presence of actinomycin D were stable, others declined in amount between 3 and 6 h after infection

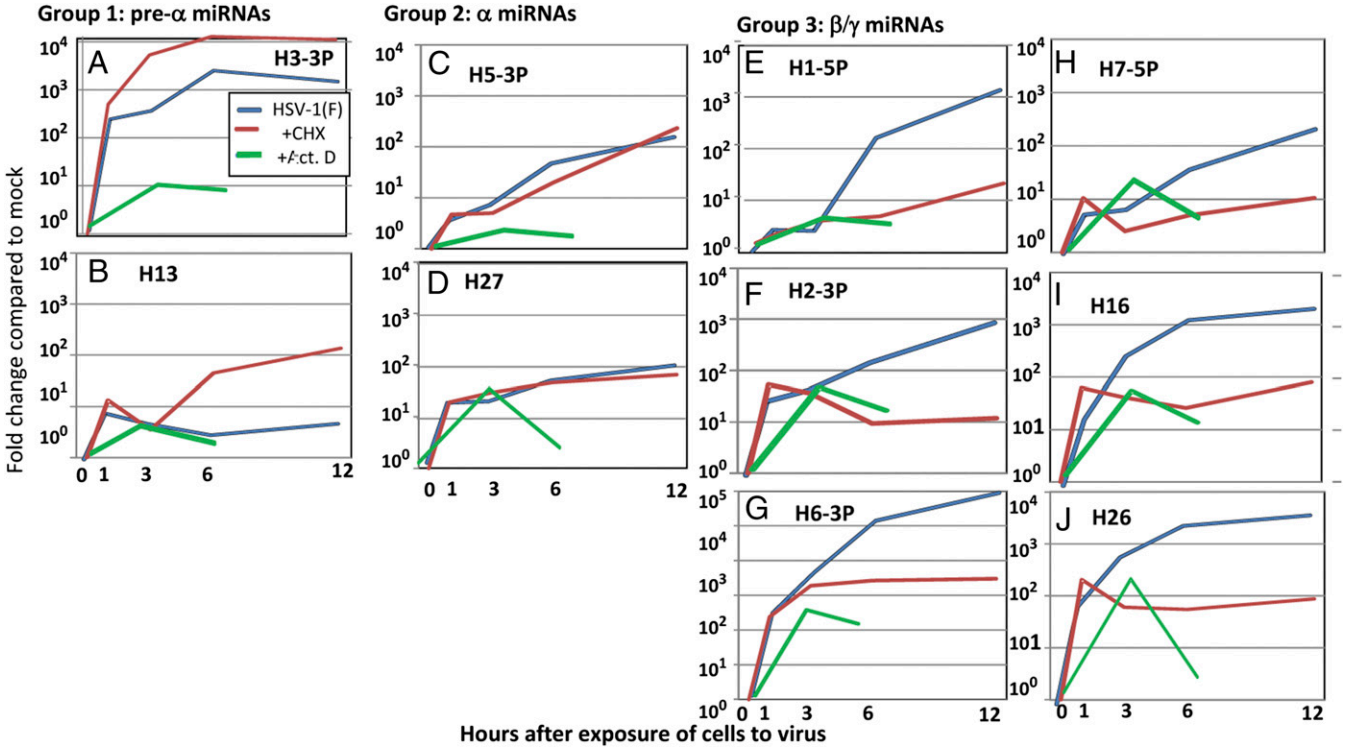

Fig. 1. Detectable HSV-1 miRNAs in infected cells. Replicate cultures of Vero cells were exposed to 10 pfu of HSV-1(F) per cell and were mock-treated or incubated in medium containing cycloheximide (100 μg/mL) collected at 0, 1, 3, 6, and 12 h after infection. A replicate set of cultures was exposed to actinomycin D (10 μg/mL) at the time and was harvested at 0, 3 and 6 h after infection. HSV-1 miRNAs normalized with respect to the cellular miRNA Let-7a are shown as fold change compared with miRNAs detected in uninfected cells (0 h). They are shown as a function of time after infection. Change folds were plotted against hours after infection. (*A* and *B*) Group 1: pre-α miRNAs. (*C* and *D*) Group 2: α miRNAs. (*E–J*) Group 3: β/γ miRNAs.

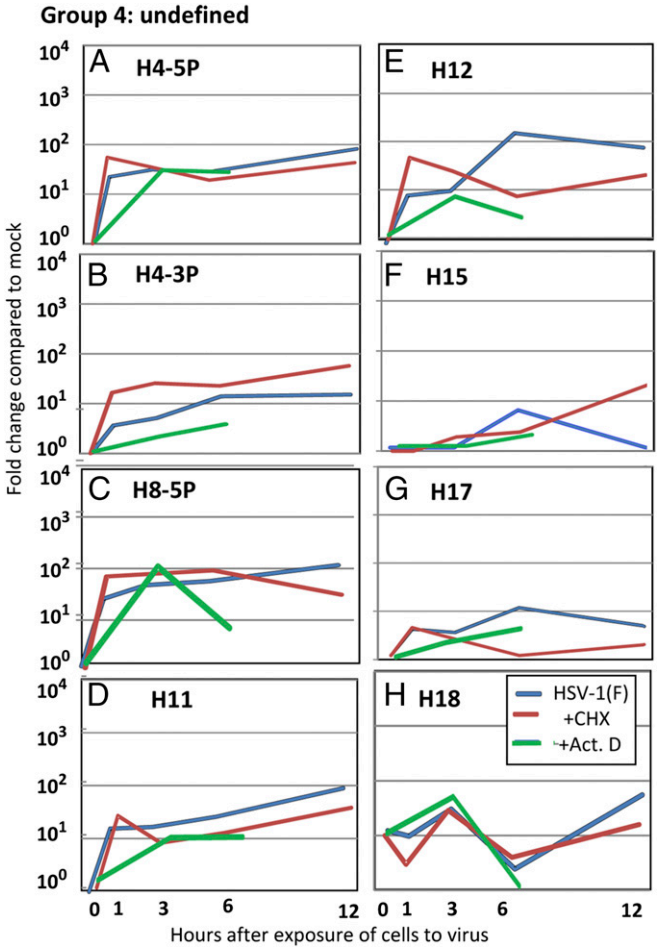

Fig. 2. (*A–H*) Group 4: undetectable miRNAs in infected cells. The experimental design is identical to that shown in Fig. 1.

(e.g., H8-5P, H27, and H26). The mechanisms underlying the apparent degradation of the RNAs are unknown.

On the basis of the patterns of their accumulation, the miRNAs could be segregated into four groups. Group 1 miRNAs (Fig. 1), consisting of H3-3P and H13, accumulated to higher amounts in the presence of cycloheximide than in medium lacking the protein synthesis inhibitor. Implicit in this finding is that one or more α proteins made after infection blocks the synthesis of these miRNAs or enhances their rate of degradation. The pattern of accumulation of these miRNAs is appropriately designated as pre-α.

Group 2 (Fig. 1), consisting of H5-3P and H27, accumulated equally well in the presence or absence of cycloheximide. Thus, the accumulation of these miRNAs does not require prior protein synthesis—a characteristic of the synthesis of α mRNAs.

Group 3 (Fig. 1) consisted of six miRNAs (H1-5P, H2-3P, H6-3P, H7-5P, H16, and H26). In each instance, the accumulation of miRNAs was lower in the presence of cycloheximide than in medium lacking the inhibitor. The synthesis of these miRNAs requires prior viral protein synthesis. The patterns of accumulation of these miRNAs resemble those of β or γ viral mRNAs.

Lastly, in group 4 we placed miRNAs (Fig. 2) whose presence in infected cells could not be authenticated because they were present at very low levels, the levels did not increase at least fivefold beyond the level observed at the start of infection, the results were not reproducible, or the amounts fluctuated excessively from one time point to the next. For example, the results shown for H11 (Fig. 2*D*) represent the highest increase observed for the interval between 1 and 12 h after infection. In repeat experiments, we observed no increase at all over the 12-h interval.

Accumulation of miRNAs in Murine Ganglia During Latency and Reactivation. In this series of experiments, 30 4-wk-old mice were inoculated by the corneal route with 10^5 pfu of HSV-1(F) per eye. Another 15 mice were kept as controls. At 30 d after inoculation, all mice were killed, and the TG were harvested. The ganglia from the 15 infected mice were processed immediately. The ganglia from the remaining 15 infected mice were incubated in medium containing anti-NGF antibody for 24 h and then processed. Analyses of the ganglia were performed as described elsewhere (14–16). Each experimental point represents the average of results obtained on six ganglia selected at random.

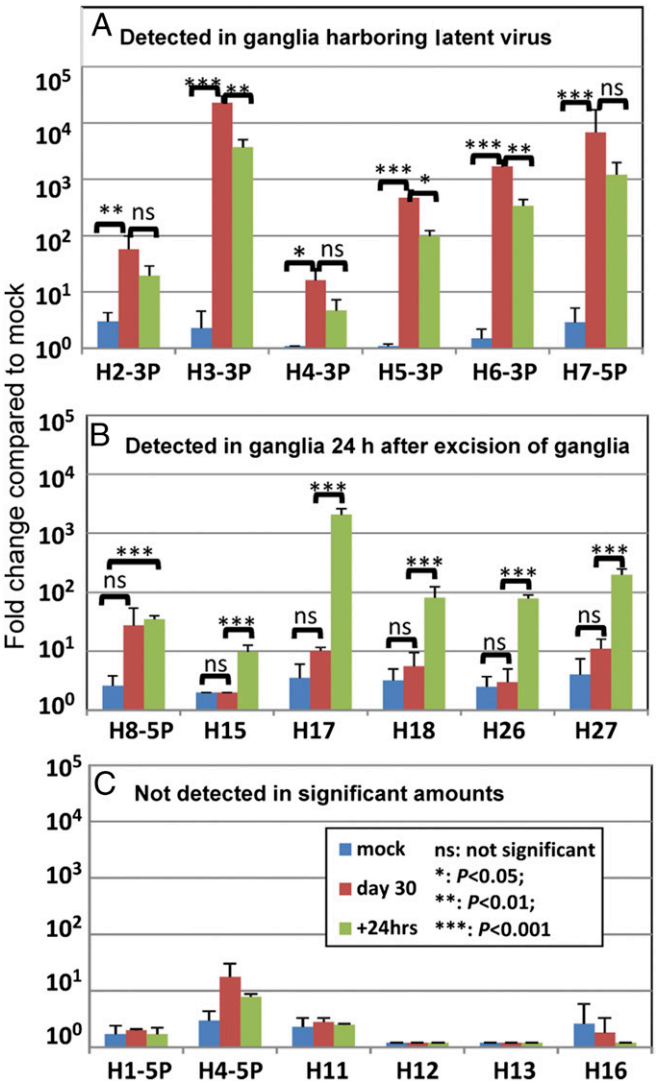

Fig. 3. HSV-1 miRNA expression in mice TG in latency and reactivation. At 30 d after inoculation, mice TG were excised and either analyzed immediately or incubated in medium containing anti-NGF antibody for 24 h. The procedures for extraction and assays were as described in *Materials and Methods*. HSV-1 miRNAs normalized by cellular miRNA Let-7a were compared with miRNAs detected in uninfected mice, presented as geometric means ± SD based on six samples per group. Student *t* test was performed to compare groups. The *P* values calculated by Student *t* test are shown. Two-tailed *P* value of 0.05 or less was considered statistically significant. (*A*) Detected in ganglia harboring latent virus. (*B*) Detected in ganglia 24 h after excision of ganglia. (*C*) Not detected in significant amounts.

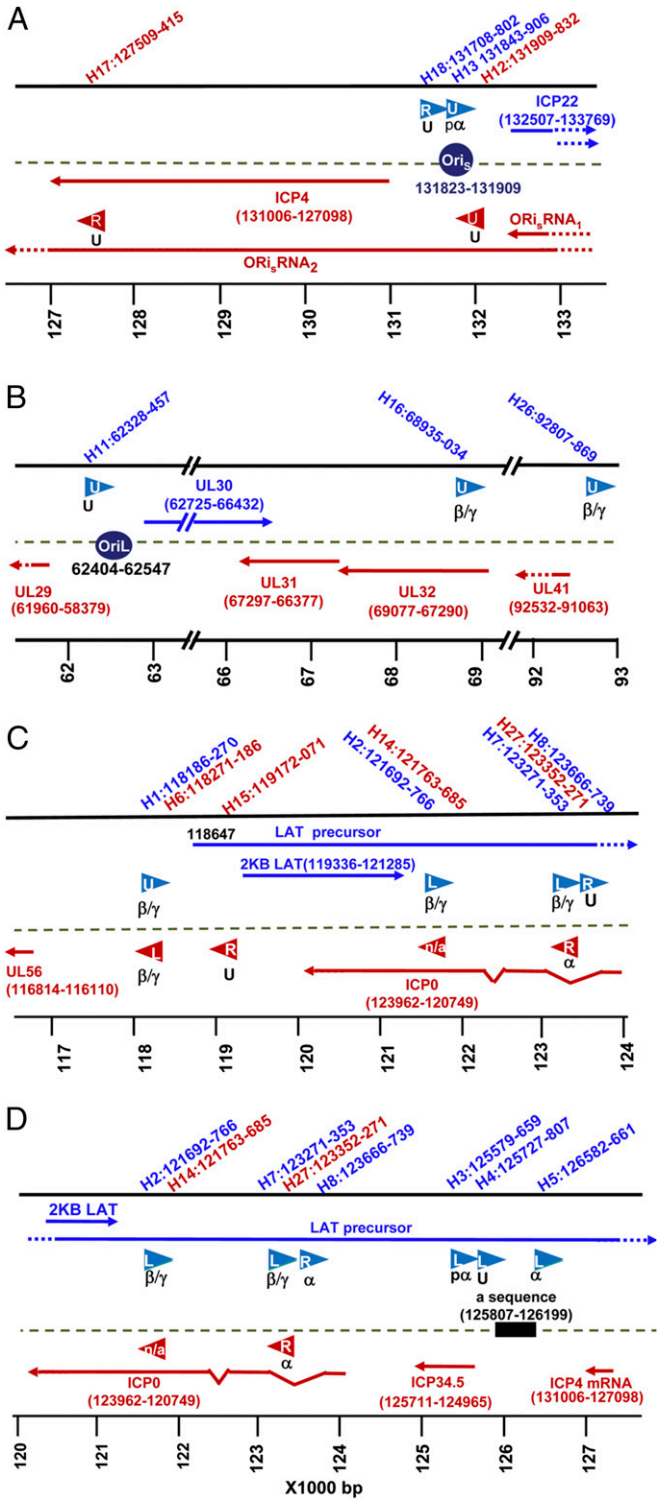

Fig. 4. Pattern of organization and expression of HSV-1 miRNAs. Shown are the locations of coding domains of relevant genes, miRNAs reported in these studies, and key *cis*-acting sites (*A*–*D*). The functions encoded in DNA strand from left to right are shown in blue above the dashed line, and the functions encoded in the strand from right to left are shown in red below the dashed line. The position of the sequences encoding the specific functions or *cis*-sites (e.g., Ori_S, Ori_L, or the a sequence) are for the HSV-1(F) strain and may be different for other HSV strains. The locations of miRNA precursors in the viral genomes are shown as arrowheads. The notations L, R, U, and n/a inside the arrowheads refer to detection of these miRNAs in mice ganglia: L, present in ganglia harboring latent virus; R, present in the ganglia incubated in medium containing anti-NGF antibody; U, undetected in ganglia; n/a, not available. Letters under arrowheads refer to miRNAs detected from lytic infected cells: pα (pre-α), α, and β/γ indicate the kinetic class of expression of viral genes; U, undetected. (*C* and *D*) For contextual completeness, the location of H14 is shown. H14 is antisense to H2. Its presence has not been verified in this study.

The data obtained from the infected ganglia were normalized as described in the legend to Fig. 3. The results (Fig. 3) were as follows.

Of the 18 miRNAs tested, 6 were present in significant amounts in ganglia harvested and processed on day 30 after infection. These were H2-3P, H3-3P, H4-3P, H5-3P, H6-3P, and H7-5P (Fig. 3*A*). At least three miRNAs in this group exhibited a significant decrease on incubation of the ganglia in medium containing anti-NGF antibody.

Analyses of miRNAs in ganglia incubated for 24 h in medium containing anti-NGF antibody revealed significant levels of accumulation of miRNAs above the levels observed in ganglia harvested on day 30. These were H8-5P, H15, H17, H18, H26, and H27 (Fig. 3*B*).

Finally, we did not detect significant accumulation of six viral miRNAs in murine ganglia. These were H1-5P, H4-5P, H11, H12, H13, and H16 (Fig. 3*C*).

Discussion

In this study, we took advantage of the results of several studies (6–12) to characterize the accumulation of 18 miRNAs encoded by HSV-1 under three conditions—i.e., in productively infected cells in culture, in ganglia harboring latent virus, and in ganglia in which latent HSV is undergoing reactivation but before the infection spreads via virions to uninfected cells. We previously described a ganglion organ culture method suitable for analyses of viral gene expression during latency and during reactivation. In these studies, TG were removed 30 d after corneal inoculation of mice and subjected to analyses immediately after excision or after incubation in medium containing antibody to NGF for 24 h—i.e., within the time frame of a single HSV-1 replicative cycle (15, 16). We reported more recently that infected HEp-2 cells export to recipient cells exosomes containing viral RNAs (14). The significance of this finding is that it enables the monitoring of the accumulation of miRNAs from the time of infection under three conditions—i.e., in untreated cells, in cells treated with cycloheximide, or in the presence of actinomycin D. The accumulation of miRNAs in the presence of cycloheximide indicates that their synthesis does not require prior protein synthesis, whereas the absence of miRNAs indicates that prior viral protein synthesis is required. The miRNAs accumulating in the presence of actinomycin D during early hours after infection provided the baseline against which we measured the accumulation of miRNAs made after infection. The results are summarized in Fig. 4 and Table 1. The significance of the results presented in this report is as follows.

(*i*) We have detected six viral miRNAs in ganglia excised 30 d after inoculation and processed immediately. These were H2-3P, H3-3P, H4-3P, H5-3P, H6-3P, and H7-5P. Our tally differs from that reported by Umbach et al. (6) in that we detected in addition H7-5P, but failed to detect H4-5P. With the exception of one study (8), which reported 10 miRNAs in ganglia, there appears to be a general agreement that the number of miRNAs in ganglia harboring latent virus is generally small, with all datasets including H6, H2, H3, and H5.

The source of the precursor of the six miRNAs that we have detected in latently infected ganglia is less clear. The 5′ boundary of LAT precursor domain (pLAT) in the HSV-1(F) strain is at nucleotide 118,647—i.e., several hundred nucleotides downstream of the H1-5P/H6-3P miRNAs. The 3′ boundary is disputed. Wagner, Stevens, and colleagues reported that the 3′ terminus is within the boundary of the L component that is at or before the a sequence or, for example, at the transcription termination of the

Table 1. Localization and properties of the miRNAs

Genomic location	HSV-1 miRNA designation	Regulation in cell culture	Expression in ganglia
Pre-pLAT			
	H1-5P	βγ	U
	H6-3P	βγ	L
	H15	U	R
pLAT			
	H2-3P	βγ	L
	H7-5P	βγ	L
	H8-5P	U	R
	H27	α	R
Post-pLAT			
	H3-3P	pα	L
	H4-3P	U	L
	H4-5P	U	U
	H5-3P	α	L
OriS-RNA2			
	H18	U	R
	H13	pα	U
	H12	U	U
ICP4			
	H17	U	R
OriL			
	H11	U	U
UL32			
	H16	βγ	U
UL41			
	H26	βγ	R

pα, pre-α; α, β, γ, kinetic class of expression of viral genes; L, detected in ganglia harboring latent virus; R, detected in ganglia incubated in medium containing anti-NGF antibody; U, uncertain or undetected.

ORF P and ORF O transcripts (2, 17). Coen, Knipe, and colleagues reported that pLAT extends beyond the L–S component junction (18). On the basis of map position of pLAT, H6 is originated from a transcript 5′ to pLAT. H2-3P and H7-5P maps indisputably within pLAT, whereas H3-3P, H4-5P, and H5-3P could have originated from pLAT, provided that pLAT extends beyond the **a** sequence (Fig. 4 *C* and *D*). The **a** sequence flanks in inverted orientations both the L and S components of HSV DNA. It consists of short sequences repeated several times in direct orientation. Direct repeat 1 of the **a** sequence is the site of cleavage of unit-length HSV DNA from concatemers (19).

A confounding issue relates to the synthesis of miRNAs in infected cells in culture (Fig. 1). pLAT accumulates late in infection. The synthesis of H6-3P, H2-3P, and H7-5P requires prior protein synthesis, suggesting that they resemble β or γ (βγ) RNA transcripts. In contrast, in infected cells in culture, H3-3P and H5-3P do not require prior protein synthesis for their expression. Specifically, the accumulation of H5-3P could not be differentiated from that of α mRNAs, whereas H3-3P accumulated to higher levels in the presence of cycloheximide than in untreated cells. These findings suggest that both miRNAs arise from a different transcript(s) than pLAT, that this transcript is not detected in ganglia because its life span is short, and that only the miRNAs accumulate over time. One implication of these findings is that the H6-3P, H3-3P, and H5-3P transcriptional units are distinct from pLAT. The putative transcriptional unit that gives rise to H1-5P or H6-3P has not been identified. It should be noted that Kramer et al. (9) reported that deletion of the LAT promoter markedly reduced expression of LAT-encoded miRNAs as well as that of H6. This finding does not alter the likelihood that H6-3P originates from an independent transcript that is affected by changes in the pLAT promoter. It is noteworthy that transcripts independent from that of pLAT and that cross the LS component junction have been reported (20).

Jurak et al. identified H1 among miRNA in ganglia harboring latent virus (8). In cell culture, H1 behaves like a viral βγ gene (Fig. 1). However, we, like others (6, 9), failed to detect H1 miRNA in ganglia. The transcribed domains of H1 and H6 partially overlap, but the mature miRNAs do not.

(*ii*) Most of the miRNAs present in ganglia harboring latent virus decrease after incubation in medium containing anti-NGF antibody, a condition that accelerates reactivation of latent virus. Concurrently, six other miRNAs have been detected in the course of reactivation of latent virus. These are H8-5P, H15, H17, H18, H26, and H27. H8-5P and H27 map within the domain of infected cell protein 0 (ICP0) intron 1. H8-5P, H15, H17, and H18 were not detected in productively infected cells (Fig. 2). ICP27 is derived from the ICP intron 1, and, as would be expected, in cell culture the synthesis of H27 was unaffected by cycloheximide. As noted in Fig. 4, H27 and H7-5P pre-miRNAs overlap, although the mature miRNAs overlap only in part. The time course and drug sensitivity of H27 suggests that it may be made concurrently with ICP0 mRNA.

H18 miRNA maps adjacent to the S-component origin of DNA synthesis, between the transcription initiation sites of ICP4 and ICP22/ICP47 within the domain encoding S component origin of DNA synthesis (OriS) RNA_2. The accumulation of H18 miRNA in infected cells was too low for the determination of the kinetics of its synthesis. OriS RNA_2 is made late after infection (21).

Lastly, three miRNAs accumulating during the reactivation phase—H15, H17, and H26—map within the domains of pLAT, ICP4, and between transcription initiation sites of U_L41, and U_L42, respectively. In cell culture, H26 miRNAs are made with βγ kinetics. We could not detect with reasonable certainty the accumulation of H15 and H17 in infected cells.

(*iii*) We were unable to detect H1-5P, H4-5P, H11, H12, H13, or H16 in ganglia harboring latent virus or in ganglia incubated for 24 h in medium containing anti-NGF antibody. We cannot exclude the possibility that their expression was transient and no longer detectable at the time of RNA extraction. Both H4-5p and H11 were not detected in infected cells or ganglia. H12 overlaps with H13, but the mature miRNAs do not overlap, and neither was detected in ganglia. In infected cells, H13 miRNA accumulated with pre-α kinetics, whereas H12 was not detected.

(*iv*) In infected cells, H1-5P and H6-3P accumulated with βγ mRNA kinetics, but only H6-3P was detected in neurons harboring latent or reactivating virus. H1-5P is antisense to H6-3P.

Several findings detailed in this report raise questions regarding the synthesis of the miRNAs.

(*i*) Three miRNAs (H4-5P, H11, and H12) did not increase significantly after exposure of cells to virus and therefore were not included in the list of miRNAs made after infection. They also were not detected in infected cells or in ganglia harboring latent or reactivating virus. H4-5P is located 5′ to the transcription-initiation site of the γ34.5 gene and extends into the DR1 repeat of the **a** sequence (22) (Fig. 4). The 5′ 65 nucleotides of H11 precursor are complementary to the 65 nucleotides at its 3′ terminus. The H12 precursor overlaps with H13, but the mature miRNAs do not overlap. The failure to detect them could reflect differences in HSV strains [HSV-1(F) vs. HSV-1(KOS) or HSV-1 (17)].

(*ii*) In an earlier publication, we noted that three miRNAs present in ganglia during the latent state decreased in amount on reactivation (15). We also reported that the miRNAs were intrinsically stable and that the decline could be interpreted as active degradation of the miRNAs. The studies reported here reinforce that conclusion. The observed decline could be interpreted in two ways. The first is that miRNAs decline ~10-fold across the board in all latently infected neurons. An alternative explanation, which we favor, is that HSV does not reactivate in

Table 2. Oligonucleotide sequence of stem-loop RT-PCR reagents for some miRNAs

miRNA	Reagent	Sequence
H11	Stem loop	5′-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC GCGTTC-3′
	Forward	5′-GTGCAGGGTCCGAGGT-3′
	Reverse	5′-GGGTGGTTAGGACAAAGTGC-3′
	Probe	5′-AGAGCCAACGCGTTC-3′ (LNA)
H15	Stem loop	5′-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC CGTGGC-3′
	Forward	5′-GTGCAGGGTCCGAGGT-3′
	Reverse	5′-TTTTTTGGCCCCGGGCCG-3′
	Probe	5′-AGAGCCAACCGTGGC-3′ (LNA)
H17	Stem loop	5′-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC CCGCCT-3′
	Forward	5′-GTGCAGGGTCCGAGGT-3′
	Reverse	5′-TTTTTTGGCGCTGGGGCG-3′
	Probe	5′-AGGCGG GTTGGCTCTGG-3′ (LNA)

all neurons harboring latent virus. If HSV reactivated in 90% of the neurons harboring latent virus, the residual miRNA levels would reflect only the neurons in which the virus did not reactivate. The mechanism by which miRNAs are degraded is unknown.

(*iii*) Several miRNAs were detected in ganglia, but not in cell culture. These included H8-5P, H4-3P, H15, H17, and H18. Of the five, four—H15, H8-5P, H17, and H18—were made in appreciable amounts in ganglia incubated in the course of reactivation of latent virus. There are two possible explanations for this finding. Foremost, it is conceivable that the population of miRNAs accumulating during the course of infection differ depending on the cell line. An alternative explanation that has not been adequately explored is based on the observation that the transcriptional program in neurons in which latent virus reactivates differs from that of productively infected cultured cells (15).

(*iv*) Of the 18 miRNAs examined in this study, H13, H1-5P, and H16 could be detected in infected cultured cells, but not in ganglia harboring latent or reactivating HSV-1. A key property of H13 is that it was detected in cells infected and maintained in the presence of cycloheximide. H13 miRNA was not detectable in untreated infected cells.

(*v*) It is intriguing that, for the most part, the miRNAs accumulating in ganglia harboring latent virus also accumulated in productively infected cells. Conversely, the miRNAs that accumulated during the 24 h after the onset of reactivation were largely absent or could not be detected in large quantities in productively infected cells. These finds are inconsistent with the hypothesis that miRNAs made during the latent state suppress viral gene expression or that those made during reactivation aid HSV in its reawakening.

(*vi*) Finally, the most critical paradox to emerge from these studies is the regulation of miRNAs and pLAT. Although LAT was described as accumulating in infected cells in low amounts at late times after infection, LAT and most of the miRNAs characteristic of neurons harboring latent virus do accumulate in productively infected cells. In these cells, the synthesis of LAT and of most miRNAs present in latently infected ganglia is characteristic of late genes—i.e., genes that require prior synthesis of viral proteins for their synthesis. Such viral effectors of accumulation and H6-3P, H2-3P, H7-5P, and LAT in productively infected cells are not made in ganglia harboring latent virus. It is of interest to explore the possibility that a neuronal host protein substitutes for the missing viral effector and constitutes the basis for the selection of sensory neurons as the site for life-long persistence of the virus.

Materials and Methods

Cells and Viruses. Vero cells were maintained in DMEM supplemented with 5% (wt/vol) newborn bovine serum. HSV-1(F) was as reported (23). The virus stocks used in these studies were purified as described (14).

Murine Model of HSV-1 Latent Infection and Reactivation. Four-week-old inbred female CBA/J mice (Jackson Labs) were infected by 10^5 pfu of HSV-1 (F) virus through corneal route as reported (15). At 30 d after infection, TG were removed and incubated in 199V containing anti-NGF antibody (Abcam) in 37 °C, plus 5% CO_2 for 24 h to induce reactivation from latency. All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of the University of Chicago.

Extraction of RNAs Enriched of Small RNAs. RNAs enriched of small RNAs were extracted from infected cells or mice TG by mirVana miRNA isolation kit from Life Technologies according to manufacturer's instructions and previous report (15).

Stem-Loop Quantitative RT-PCR. A total of 18 HSV-1 miRNAs were tested in this study by real-time PCR after stem-loop reverse transcription as described in detail in ref. 24. Cellular miRNA Let-7a was tested as internal control. RNAs enriched of small RNAs were reverse-transcribed by specific stem-loop primer using the SuperScript III reverse transcriptase kit from Life Technologies (40 ng of RNA for each tested miRNA). Stem-loop reverse-transcription primers and Taqman assays for H4-5P, H4-3P, H7-5P, H8-5P, H12, H13, H16, H18, H26, and H27 were purchased from Life Technologies. Taqman assays of Let-7a, H3-3P, H5-3P, and H6-3P were custom-synthesized by Life Technologies on the basis of sequences reported by Umbach et al. (6). H1-5P and H2-3P were tested by the SYBR green system using primer sequences for stem-loop reverse transcription and quantitative PCR as described by Umbach et al. (6). Stem-loop reverse-transcription primers, quantitative RT-PCR primers, and locked nucleic acid (LNA) probes for H11, H15, and H17 were designed and purchased from IDT, with sequences as listed in Table 2. All of HSV-1 miRNAs expressions levels were normalized to cellular miRNA Let-7a and presented as fold increase compared with levels in uninfected cells or the lowest uninfected mouse ganglion.

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