JOURNAL OF VIROLOGY, Feb. 2009, p. 1433–1442 Vol. 83, No. 3
0022-538X/09/$08.00+0 doi:10.1128/JVI.01723-08

Novel Less-Abundant Viral MicroRNAs Encoded by Herpes Simplex Virus 2 Latency-Associated Transcript and Their Roles in Regulating ICP34.5 and ICP0 mRNAs▽

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Received 13 August 2008/Accepted 11 November 2008

We recently identified an acutely and latently expressed viral microRNA (miRNA), miR-I, encoded by herpes simplex virus 2 (HSV-2) latency-associated transcript (LAT) through small RNA cloning and two miRNAs encoded by HSV-1 LAT through prediction. We now report the use of high-throughput sequencing technology to identify two additional relatively less-abundant viral miRNAs, miR-II and miR-III, encoded by HSV-2 LAT exon 2. miR-II includes two miRNAs, miR-II-5p and miR-II-3p, which are processed from the same miRNA precursor. miR-II and miR-III map antisense to the 5′ untranslated region of ICP34.5 and to the coding region of ICP0 exon 3, respectively. These novel miRNAs are conserved in different HSV-2 strains, and their presence in infected- and transfected-cell cultures was confirmed by Northern hybridization. All three HSV-2 LAT-encoded miRNAs map to genome locations similar to those of three out of four identified HSV-1 LAT-encoded miRNAs, but the sequences of these miRNAs are not conserved. The expression of LAT-encoded miRNAs is negatively regulated by ICP4, the major viral transactivator. We further show that, similar to miR-I, miR-II is able to efficiently silence the expression of ICP34.5, a key viral neurovirulence factor, and that miR-III is able to silence the expression of ICP0, a key viral transactivator. All these data suggest that LAT sequences likely contribute to HSV latency and reactivation through tight control of these LAT-encoded miRNAs and their viral targets.

Herpes simplex virus 1 (HSV-1) and HSV-2 are closely related herpesviruses. HSV-1 typically infects the facial region and establishes a lifelong latent infection in sensory neurons of the trigeminal ganglia, while HSV-2 typically infects the genital region and establishes a lifelong latent infection in sensory neurons of the sacral dorsal root ganglia. Periodically, either virus may reactivate to cause symptomatic or asymptomatic recurrences in the area served by these sensory neurons. HSV-2 and HSV-1 have similar latent transcription patterns, in which the latency-associated transcript (LAT) is transcribed from within the genomic long repeats. In contrast to other viral promoters, the LAT promoter is highly active during latency, and LAT is the only viral gene product that is readily detectable during latency (39). HSV-1 LAT expression is inhibited by ICP4, the major viral transactivator required for most post-α gene expression (9, 12, 24), through an ICP4 binding site near the LAT transcription initiation site (14). The LAT introns (~2.2 kb in HSV-2 and ~2 kb and 1.4 kb in HSV-1), which overlap the ICP0 transcript in an antisense direction, are much more abundant and stable than the ~8.5-kbp primary LAT transcript (13), which overlaps both the ICP0 and ICP34.5 transcripts in an antisense direction. ICP0 can transactivate a number of viral and host genes and is essential for HSV reactivation (4, 5, 18, 19). ICP34.5, a key viral neurovirulence factor, is a protein kinase R inhibitor and is required for efficient viral replication in neurons in vivo (2, 6, 7, 49).

The LATs play an important role in HSV latency and reactivation. Deletion of the LAT promoter in both HSV-1 and HSV-2 reduces the efficiency of reactivation (25, 28, 35, 38, 45, 47, 50). The hypothesized mechanisms by which LAT could act include inhibition of replication during acute infection of neurons via an antisense mechanism (13, 37, 39), thus promoting neuronal survival. The HSV-1 LAT is currently believed to act at least in part by increasing the establishment or maintenance of latency (35, 45), likely via an effect on the survival of acutely infected neurons (44). Animals infected with an HSV-1 LAT deletion mutant virus are more likely to have apoptotic neurons during the acute infection (32, 46).

MicroRNAs (miRNAs) are a family of 21- to 24-nucleotide (nt) noncoding RNAs that regulate gene expression based on sequence similarity to their target (2, 11, 22). Recently, we reported an acutely and latently expressed miRNA encoded by HSV-2 LAT that inhibits ICP34.5 expression and two HSV-1 LAT-encoded miRNAs that also map antisense to the ICP34.5 region (40). Cui et al. reported an HSV-1 miRNA that mapped upstream of the LAT (10), and Umbach et al. recently reported four HSV-1 LAT-encoded miRNAs (48). In the present study, we identify two additional relatively less-abundant novel virally encoded miRNAs in HSV-2 LAT exon 2 by using 454 high-throughput (HTP) sequencing technology. We further show that the expression of these LAT-encoded miRNAs is negatively regulated by ICP4 and that the novel viral miRNAs can inhibit expression of ICP34.5 and ICP0.

MATERIALS AND METHODS

Cells, viruses, and antibodies. HSV-2 strain HG52 (GenBank accession no. NC_001798) and HSV-1 strain 17syn+ (GenBank accession no. NC_001806) genome sequences were used as reference sequences. Vero, HEK 293, HeLa, and U2OS cell lines were obtained from ATCC. HSV-2 strain 333 was obtained from Gary Hayward (Johns Hopkins University, MD). HSV-2 strain HG52 was

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▽ Published ahead of print on 19 November 2008.

obtained from Larry Stanberry (Columbia University, NY). HSV-1 ICP4-binding site mutant virus R7530 and it rescuant virus, R7531, were obtained from Bernard Roizman (University of Chicago, IL). Rabbit polyclonal anti-HSV-2 ICP34.5 antibody was raised against synthetic peptides corresponding to the N terminus of HSV-2 ICP34.5 (40). Anti-β-tubulin is from BD Bioscience (CA). Detection of HSV-2 ICP34.5 by Western blotting was performed as described previously (40).

Oligonucleotide probes and RNA oligonucleotides. Oligonucleotide probes for miR-I, the miR-I homolog (miR-H3), miR-LAT-ICP34.5 (miR-H4-3p), and U6 snRNA were described previously (40). Oligonucleotide probes for miR-II-5p (oST486; GCATGCGTGCCGAGTGAACTC), miR-II-3p (oST485; TGAGTT CGCTAGGCAAGCACGG), miR-III (oST525; TCGCGCATGACCCAGGCT CAGA), the predicted antisense strand of miR-III (oST526; AGTCTGGGCC GGGCAGGCGCG), and the miR-H5 homolog (predicted miR-IV) (oST533; GCGGGGGGTCTGGGGCTCTGAC) and its predicted antisense strand (oST534; AGGTCAGGTGGCCCGAGCCCCC) were chemically synthesized (Invitrogen, CA). RNA oligonucleotides corresponding to the sequences of HSV-2 miR-II-5p (oST523; AGAGUUCACUCGGACGCAUGC) and miR-II-3p (oST524; CCGUCUUGCCUAGCGAACUCA) were chemically synthesized (Dharmacon, CO) and annealed in 1× annealing buffer (Dharmacon, CO) to make the miR-II duplex. Similarly, RNA oligonucleotides corresponding to the sequences of HSV-2 miR-III (oST527; UCUGAGCCUGGGUCAUGCG CGA) and the predicted antisense strand (oST528; CGCGCCUGCCCGGCCC AGACU) were also synthesized by Dharmacon and annealed to make the miR-III duplex. 2′-*O*-Methyl-modified RNA oligonucleotides including the miR-II-5p inhibitor (oST504; mGmUmGmCmAmUmGmCmGmUmGmCmCmGmAmG mUmGmAmAmCmUmCmU), miR-III inhibitor (oST529; mGmUmCmGmCm GmCmAmUmGmAmCmCmCmAmGmGmCmUmCmAmGmAmC), and NS miRNA inhibitor (completely complementary to HSV-1 miR-LAT-ICP34.5 as described previously [40]) were also synthesized by Dharmacon.

Plasmids, transfection, and dual luciferase assay. pSSK, pCMV-SSK, pSSB, pAvrII-Sap, pAvrII-AluI, and pRL-Ts were described before (40). The pmiR-II-cluster was cloned by inserting a PCR-amplified HSV-2 ICP34.5 region (with PCR primers oST499 [TGTTCGCCCACTCTGCGTCGTCGT] and oST500 [TCCTGCCGCCGCCCCTTAAGAG]) into a pFlag vector (Sigma, MO). pICP34.5 was cloned by first inserting a PCR-amplified HSV-1 ICP34.5 region (with PCR primers oST487 [CGCGCGGCCCTTTAAAGCGGTG] and oST488 [AGACCCAGGCCGCCTCGGGTGTAAC]) into a pCR4 Topo clone vector (Invitrogen, CA) and then subcloning into the pFlag vector (Sigma, MO) EcoRI site. pLAT2-Luc1 and pLAT2-Luc1R were constructed by inserting the NotI fragment (including the HSV-2 LAT promoter) from pSSB into the pFlag vector and then subcloning into the pGl3-Basic luciferase vector (Promega, WI) BamHI and HindIII sites. pLAT1-Luc3 was cloned by inserting a PCR-amplified HSV-1 LAT promoter region using primers (oST480 [CGGCCGGCTACCGAGACCG AACA] and oST483 [GCTGCAGGGGGGCCCGGAGA]) into a pCR2 Topo cloning vector (Invitrogen, CA) and then subcloning into the pGl3 luciferase vector BamHI and HindIII sites. Similarly, a shorter PCR fragment (with primers oST480 and oST484 [ACGGCCGCGCCCCCGCTTTTAT]) was inserted into the pGL3-Basic vector to make pLAT1-Luc4. pSSK and pAvrII-SapI were used as PCR templates to amplify HSV-2 LAT and HSV-1 LAT regions, respectively. pICP4 (HSV-2 ICP4 coding region under the control of the cytomegalovirus [CMV] immediate early [IE] promoter) was obtained from Kening Wang and Jeffrey Cohen (NIH, Bethesda, MD). Plasmids and RNA oligonucleotides were transfected with Lipofectamine 2000 (Invitrogen, CA) into HEK 293 cells or U2OS cells. The dual luciferase assay was performed with a dual luciferase assay kit (Promega, WI) as described previously (42).

454 HTP sequencing of small RNA libraries from LAT-transfected cells and detection of miRNAs by Northern blotting. Small RNA libraries from HEK 293 cells transfected with pSSK or pCMV-LAT were constructed previously (40). pSSK and pCMV-SSK libraries were reamplified with A and B sequence-adapted PCR primers to enable them to be sequenced by a 454 genome sequencer (30). The above two libraries shared one run with 14 other samples in a 454 genome sequencer (Roche, MA). 454 sequencing was performed by Cogenics Inc. (NC). Viral miRNAs were detected with ^{32}P-labeled specific primers by Northern blotting as described previously (41).

Detection of virally encoded miRNAs in wild-type and LAT mutant virus-infected cells by real-time PCR. Vero cells (3×10^6) were seeded in six-well plates in triplicate 6 h before infection with wild-type HSV-2 strain 333 or NotI-SalI (an HSV-2 LAT promoter deletion mutant virus) at a multiplicity of infection (MOI) of 3. Total RNA was prepared with Trizol (Invitrogen, CA) at 0, 3, 6, 9, 14, and 18 hours postinfection (hpi). Fifty nanograms of total RNAs was used in each miRNA reverse transcription (RT) reaction. miR-I detection by real-time PCR was previous described (40). miR-II was reverse transcribed with 1 nM RT primer (oST550; GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACGCATGC) and then detected with 1.2 μM forward primer (oST549; CACTGGAGAGTTCACTCGGCAC), 0.6 μM of the previously described (40) backward primer (oST408; GTGCAGGGTCCGAGGT), and 0.1 μM TaqMan probe (oST551; 6-carboxyfluorescein [FAM]-TGGATAC GACGCATGCG-MGB). miR-III was reverse transcribed with 1 nM RT primer (oST553; GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACTCGCGC) and detected with 1.2 μM forward primer (oST552; CACTG GTCTGAGCCTGGGTCAT), 0.6 μM backward primer (oST408), and 0.1 μM TaqMan probe (oST554; FAM-TGGATACGACTCGCGCA-MGB). rRNA, used as a loading control, was quantified by real-time PCR with rRNA control reagents (Applied Biosystems, CA). RNA oligonucleotides oST523 and oST527 (described above) were used as standards for the real-time PCR. The real-time PCR conditions were as previously described (40). To detect HSV-1 miR-LAT-ICP34.5 (miR-H4-3p), 3×10^6 Vero cells were seeded in six-well plates in triplicate 6 h before infection with R7530 (ORF-O, P ICP4-binding site mutant) or R7531 (a rescuant virus of 7530). Detection of miR-LAT-ICP34.5 (miR-H4-3p) was performed using a modification of the previously described assay (48), with oST408 as the backward primer and 1 nM RT primer in the RT reaction. The real-time PCRs were performed with an ABI 7900 real-time PCR machine (Applied Biosystems, CA).

Determination of the transcription start sites of HSV-2 ICP34.5 by 5′ RACE. Vero cells were infected with HSV-2 strain 333 at a MOI of 3. Total RNA was prepared at 18 hpi using Trizol (Invitrogen, CA). Ten micrograms of the total RNA was used to determine the transcription start site of HSV-2 ICP34.5 by 5′ rapid amplification of cDNA ends (RACE) with a RLM RACE kit according to the manufacturer's instructions (Applied Biosystems, CA). Primers oST505 (C TCGGCGTAGGCCCGGA) and oST506 (CGGCTGGGCTCGGCGTA) were used as gene-specific primers. The nested PCR products were separated on a 2% agarose gel, gel purified, ligated to a pCR4 Topo clone vector (Invitrogen, CA), transformed into Topo 10 cells (Invitrogen, CA), and sequenced.

Detection of HSV-2 ICP0 by real-time PCR. An HSV-2 ICP0 real-time PCR system with primers (oST530 [GTCCGGTGGACGCGCA] and oST531 [TGCCC GGCCCAGACTCTGT]) flanking the miR-III site and a TaqMan probe (oST532; FAM-GCGCATGACCCAGGCTCAGA-6-carboxytetramethylrhodamine) overlapping the miR-III cutting site was set up to detect ICP0 with a One Step RT-PCR kit (Applied Biosystems, CA). Detection of HSV-2 thymidine kinase (TK) was by real-time PCR as described previously (40).

RESULTS

Discovery of novel less-abundant viral miRNA sequences through 454 HTP sequencing technology. We reported an abundantly expressed miRNA encoded by HSV-2 LAT sequences based on manual sequencing of two small RNA libraries constructed from LAT plasmid-transfected cells (40). To uncover potential low-abundance LAT-encoded miRNAs, we used 454 HTP sequencing technology to sequence these two previously constructed small RNA libraries. Besides the highly abundant miR-I, HTP sequencing also revealed multiple copies of four 21- to 23-nt viral sequences (miR-I-5p, miR-II-5p, miR-II-3p, and miR-III) that were not found by manual sequencing previously (Table 1). These four novel potential viral miRNA sequences all map to LAT exon 2 and are able to fold into stem-loop secondary structures with their adjacent sequences as illustrated in Fig. 1A. One very-low-abundance viral miRNA sequence identified by HTP sequencing was the antisense strand of the previously identified miR-I and thus was named miR-I-5p. The ratio of the frequency of miR-I-5p to that of miR-I is approximately 1:40. The sequence of miR-I-5p is exactly the same as previously predicted. miR-I-5p is below the detection limit of Northern hybridization in HSV-2-infected cells or LAT plasmid-transfected cells (40), suggesting that HTP sequencing can pick up low-abundance miRNAs that are not detectable by Northern blotting. Two other miRNA-like sequences are likely processed from the same stem-loop miRNA precursor and thus were named miR-II-5p and miR-

TABLE 1. New HSV-2 LAT-encoded miRNAs identified by 454 HTP sequencing

Viral miRNA[a]	Frequency[b] for:		
	pSSK library[c]	pCMV-SSK library[c]	Total
miR-I[d]	34	44	78
miR-I-5p	0	2	2
miR-II-5p	3	3	6
miR-II-3p	0	4	4
miR-III	3	5	8

[a] See Fig. 1 for the sequences of these miRNAs.
[b] Total number of times the miRNA-like sequences appeared in the clones sequenced.
[c] Small RNA libraries from pSSK- or pCMV-SSK-transfected HEK 293 cells were constructed as described previously (40). The qualified 454 sequencing reads were approximately 16,000 for the pSSK library and 19,000 for the pCMV-SSK library, respectively.
[d] miR-I has been identified and described before (40).

II-3p. miR-II-5p and miR-II-3p map approximately 160 bp downstream of miR-I, in an antisense orientation to the potential 5′ untranslated region (UTR) of the ICP34.5 transcript. miR-II-3p is approximately 76% homologous to HSV-1 miR-LAT-ICP34.5, identified previously (and also named as miR-H4 by Umbach et al. [48]). Another interesting viral miRNA-like sequence (miR-III) identified in both libraries maps antisense to the coding region of ICP0 exon 3. The frequencies of miR-II and miR-III were approximately 10 to 20 times lower than that of miR-I.

Detection of miR-II-5p and miR-II-3p by Northern hybridization in transfected and infected cells. To confirm the presence of miR-II, we first used Northern hybridization of total RNAs from transfected HEK 293 cells (Fig. 1B). We constructed a eukaryotic expression plasmid, pmiR-II-cluster, that encodes miR-II and adjacent sequences under the control of the CMV IE promoter. The pre-miRNA of miR-II-5p and miR-II-3p was detected with both 5′- and 3′-specific probes, further indicating that miR-II-5p and miR-II-3p are processed from the same pre-miRNA. Both mature miR-II-5p and mature miR-II-3p were detected with specific probes in cells transfected with pmiR-II-cluster, suggesting that it is likely that both strands of miR-II can be assembled into an RNA interference-induced silencing complex (RISC) in transfected cells. We then performed Northern hybridization on total RNAs from Vero cells infected with HSV-2. Pre-miRNAs of miR-II-5p and miR-II-3p were detected by both 3′ and 5′ probes in cells infected with HSV-2 strains 333 and HG52 (Fig. 1C). Only the mature miR-II-5p gave a specific signal (which was very weak) when detected with the 5′ probe, although miR-I could be easily detected after stripping (Fig. 1C). These data suggested that, compared to miR-I, miR-II-5p and miR-II-3p are less efficiently processed and assembled into the RISC in infected-cell cultures.

Detection of miR-III by Northern hybridization in transfected and infected cells. We used Northern hybridization of total RNAs from transfected HEK 293 cells to confirm miR-III. Mature miR-III (but not the predicted antisense strand of miR-III) was detected with a miR-III-specific probe in cells transfected with both pSSK and pCMV-SSK (Fig. 1D). We then performed Northern hybridization on total RNAs from infected Vero cells. miR-III was detected by the miR-III-specific probe in both HSV-2 strain 333- and HG52-infected cells (Fig. 1E). However, the predicted antisense strand of miR-III was not detected with a probe for the antisense strand of miR-III, indicating that miR-III is assembled into RISC and that the strand complementary to miR-III is likely degraded after unwinding.

Time course of miR-I, -II, and -III expression in HSV-2 wild-type and LAT promoter-deleted mutant virus-infected cells. We showed previously that the LAT promoter is not the only promoter for miR-I expression in an infected-cell culture, although the majority of miR-I is transcribed by the LAT promoter in vivo (40). Here, we studied the expression kinetics of the three LAT-encoded miRNAs in wild-type and LAT promoter-deleted (NotI-SalI) mutant virus-infected cells (Fig. 2). Similar to Umbach et al's observation with the HSV-1 homolog (48), there was a low-level miR-III signal in mock-infected Vero cells (0 hpi). The quantities of all three LAT-encoded miRNAs increased as the infections progressed. Both miR-I and miR-II were detectable as early as 3 hpi and in cells infected with the LAT promoter deletion mutant, implying that promoters other than the LAT promoter (a late promoter in infected-cell cultures) contribute to miR-I and miR-II expression. miR-III was detected only after 9 hpi in wild-type HSV-2-infected cells. miR-III was expressed to a higher level in wild-type HSV-2-infected cells than in LAT promoter deletion mutant-infected cells at all time points. miR-III was detectable late in infection (14 and 18 hpi) in LAT promoter deletion mutant-infected cells, possibly as a result of read-through transcription at late times in infection.

The LAT miRNA cluster is negatively regulated by ICP4. We previously showed that miR-I is expressed by the LAT promoter during latency but is also expressed by other promoters, particularly including the putative ORF-O, P promoter, during acute infection (40). HSV-1 ICP4, a key viral transactivator, inhibits both LAT and ORF-O, P expression by binding to ICP4-binding sites near the transcription start sites (14, 26, 27). Therefore, ICP4 is likely directly involved in the transcriptional regulation of LAT-encoded miRNAs. To test whether HSV-2 ICP4 functions similarly in controlling HSV-2 LAT and HSV-2 ORF-O, P promoter activity, we first constructed an HSV-2 LAT promoter reporter which contains the LAT promoter and 264 bp of downstream sequences (after the TATA box) upstream of a firefly luciferase reporter gene (Fig. 3A). HSV-1 LAT promoter reporters with or without the ICP4-binding site sequences were also constructed as controls. The reporter plasmids were cotransfected with an HSV-2 ICP4 expression plasmid and a *Renilla* luciferase plasmid into HEK 293 cells. Compared to the LAT promoter reporter, a reverse direction version of the HSV-2 LAT promoter reporter showed only a very low level of firefly luciferase activity, indicating that LAT promoter activity is directional in transfected nonneuronal cells (Fig. 3A). HSV-2 ICP4 reduced HSV-2 LAT promoter reporter activity by approximately 11-fold but had no effect on the cotransfected *Renilla* luciferase activity or pGL3-Basic (null vector), indicating that, similar to HSV-1 ICP4, HSV-2 ICP4 can inhibit LAT expression (Fig. 3A). Interestingly, HSV-2 ICP4 can substitute for HSV-1 ICP4 in suppressing the HSV-1 LAT promoter reporter that contains the HSV-1 ICP4-binding site (Fig. 3A). Deletion of the HSV-1

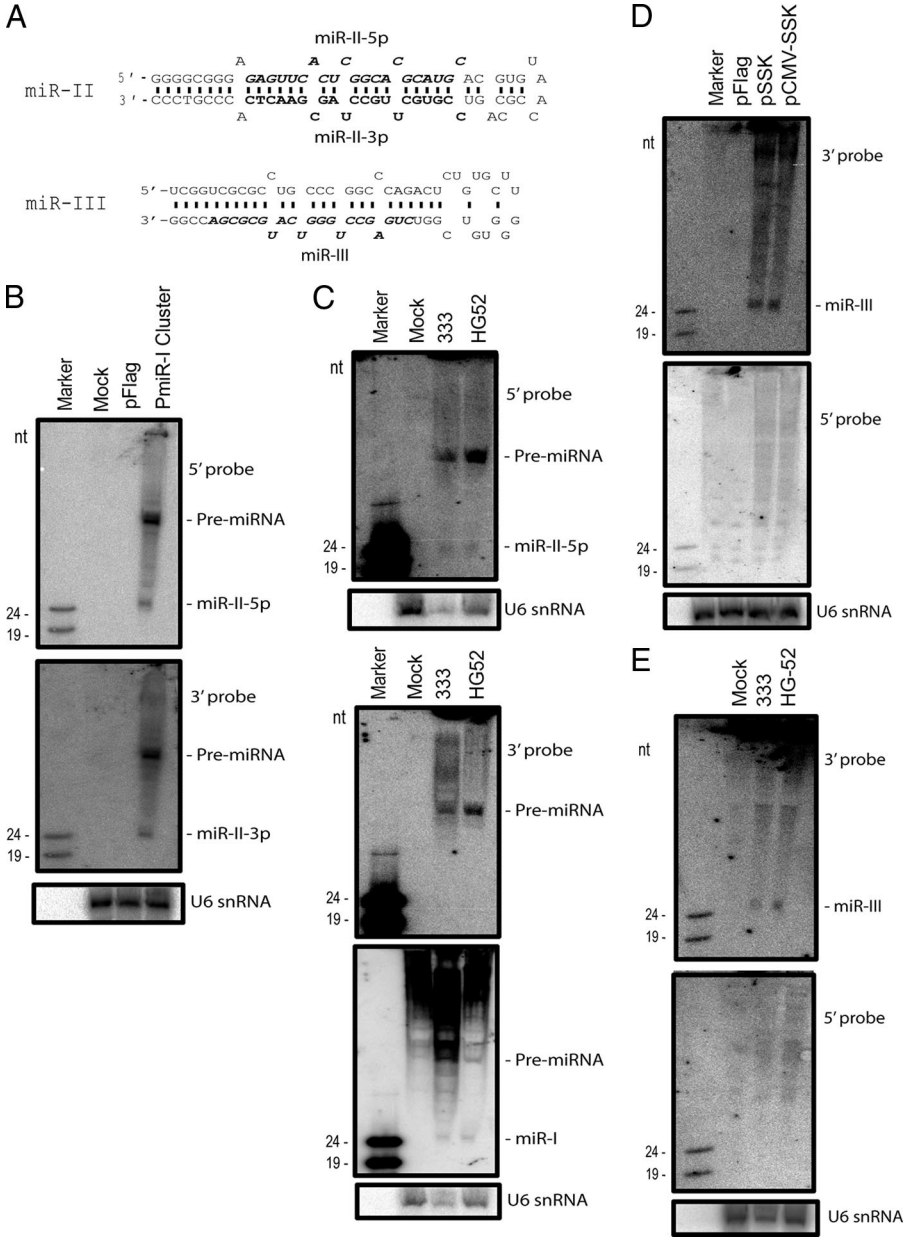

FIG. 1. Identification of HSV-2 LAT-encoded less-abundant miRNAs. (A) Diagram of the predicted secondary structures of HSV-2 miR-II and miR-III. The HSV-2 mature miR-II-5p (in boldface italics), miR-II-3p (in boldface), and miR-III (in boldface italics) were identified by HTP sequencing of two small RNA cloning libraries and map to HSV-2 LAT exon 2 (nt 386 to 366 and 126846 to 126884, nt 349 to 329 and 126901 to 126921, and nt 4273 to 4253 and 122977 to 122997, respectively). The RNA secondary structures were predicted by M-Fold. (B) HSV-2 miR-II-5p and miR-II-3p detection by Northern blotting in HEK 293 cells transfected with a plasmid containing the miR-II flanking sequences. Total RNAs from HEK 293 cells transfected with or without plasmids pmiR-II-cluster and pFlag (an empty vector) were hybridized with a ^{32}P-labeled oligonucleotide probe for miR-II-5p (top). The same membrane was stripped and reprobed with an oligonucleotide probe for miR-II-3p (middle) and a probe for U6 snRNA. (C) HSV-2 miR-II-5p and miR-II-3p detection by Northern blotting in Vero cells infected with HSV-2. Total RNAs from uninfected Vero cells and Vero cells infected with HSV-2 strains 333 and HG52 were hybridized with miR-II-5p, the miR-II-3p probe, and the U6 snRNA probe. The miR-II precursor could be detected in both HSV-2 strain 333- and HG52-infected cells with a probe antisense to miR-II-5p (top) or a probe antisense to miR-II-3p (middle). Total RNAs were also hybridized with an HSV-2 miR-I probe to serve as an additional internal control (bottom). (D) HSV-2 miR-III detection by Northern blotting in 293 cells transfected with plasmids containing the full-length LAT gene. Total RNAs from HEK 293 cells transfected with or without plasmids pSSK, pCMV-SSK, or pFlag were hybridized with a ^{32}P-labeled oligonucleotide probe for miR-III. pSSK contains the LAT sequences and its promoter sequences. pCMV-SSK is a mutant plasmid expressing LAT under the control of the CMV IE promoter. The same membrane was stripped and reprobed with an oligonucleotide probe for the predicted antisense strand of miR-III and a probe for U6 snRNA. (E) HSV-2 miR-II-5p and miR-II-3p detection by Northern blotting in Vero cells infected with HSV-2. Vero cells were infected with HSV-2 strain 333 or HG52 or were not infected. Total RNAs were hybridized with the miR-III probe, the predicted antisense strand of the miR-III probe, and the U6 snRNA probe.

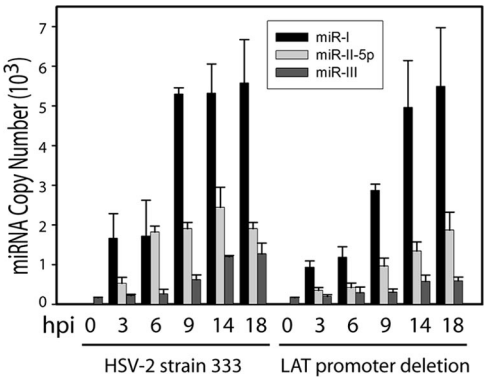

FIG. 2. Time course of LAT-encoded miRNA expression in LAT mutant virus- and wild-type virus-infected cells. Vero cells were infected in triplicate with HSV-2 strain 333 wild-type virus or a mutant in which the LAT promoter (NotI-SalI sequences) was deleted. Total RNA was prepared at 0, 3, 6, 9, 14, and 18 hpi. Fifty nanograms of total RNA was used in specific real-time PCR systems to quantify miR-I, miR-II, and miR-III at each time point for each virus.

ICP4-binding site relieved the suppression of LAT promoter activity by ICP4 (Fig. 3A), further indicating that the suppression of the LAT promoter by ICP4 is sequence specific.

To test whether ICP4 suppresses expression of LAT-encoded miRNAs, we performed Northern hybridization on total RNAs from cells transfected with LAT plasmids (pSSK and pPstI-HincII), with or without the ICP4 expression plasmid, to detect LAT-encoded miRNAs. pSSK contains the LAT sequences and its promoter sequences. pPstI-HincII contains partial LAT sequences but not the LAT promoter sequences (40). HSV-2 ICP4 inhibited the expression of LAT-encoded miRNAs, including miR-I, miR-II, and miR-III, when cotransfected with pSSK (Fig. 3B). ICP4 also inhibited miR-I expression when cotransfected with pPstI-HincII, which contains downstream sequences but not the LAT promoter sequences. miR-II-5p was barely detectable in cells transfected with pPstI-HincII, further suggesting that miR-II is a low-abundance viral miRNA. As expected, miR-III was not detected in pPstI-

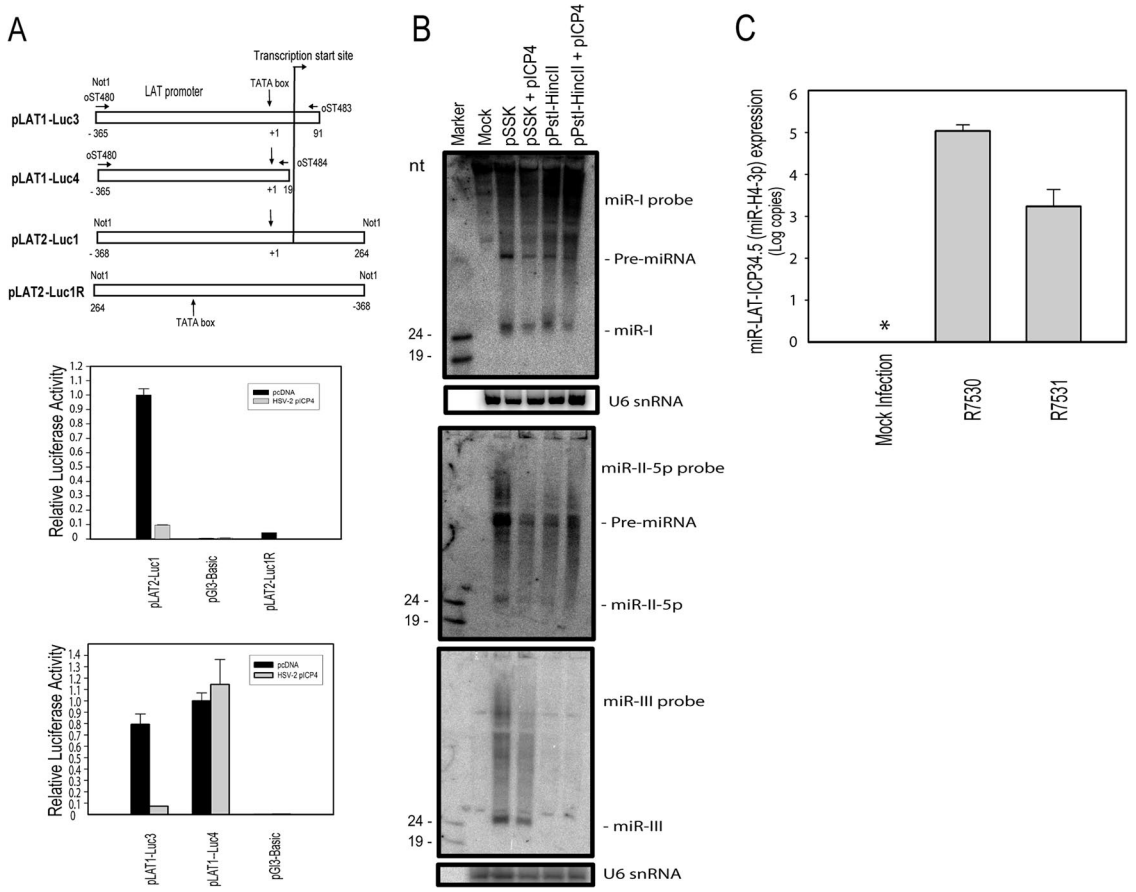

FIG. 3. ICP4 downregulates LAT-encoded miRNA expression. (A) HSV-2 ICP4 reduces LAT reporter activity. The schematic diagram illustrates the construction of HSV-1 and HSV-2 luciferase reporters. (Middle) pLAT2-Luc1, pRL-Ts (*Renilla* luciferase control), and pGl3-Basic were cotransfected with the pLAT2-ICP4 or pcDNA3 vector in HEK 293 cells. pLAT2-Luc1R was also transfected as an additional negative control. (Bottom) pLAT1-Luc3, pLAT1-Luc4 (with deletion of ICP4-binding sites), pRL-Ts, and pGl3-Basic were cotransfected with HSV-2 ICP4 or pcDNA in HEK 293 cells. Firefly luciferase activity readings were normalized with *Renilla* luciferase activity. (B) Detection of HSV-2 LAT-encoded miRNAs in cells transfected with plasmids containing LAT promoter sequences, ORF-O, P promoter sequences, and the ICP4 gene. pSSK and pPst1-HincII were cotransfected with pICP4 or pcDNA 3 into HEK 293 cells. Total RNAs were hybridized with ^{32}P-labeled probes for miR-I, miR-II-5p, miR-III, and U6 snRNA after stripping. (C) An HSV-1 mutant virus with an ORF-O, P ICP4-binding site mutation expresses more miR-LAT-ICP34.5 in infected cells. Vero cells were infected with R7530 (ORF-O, P ICP4-binding site mutant) or R7531 (rescuant virus of R7530). miR-LAT-ICP34.5 (miR-H4-3p) expression was analyzed by real-time PCR. The asterisk indicates that all mock infection controls were below the assay detection limit of ~1,000 copies.

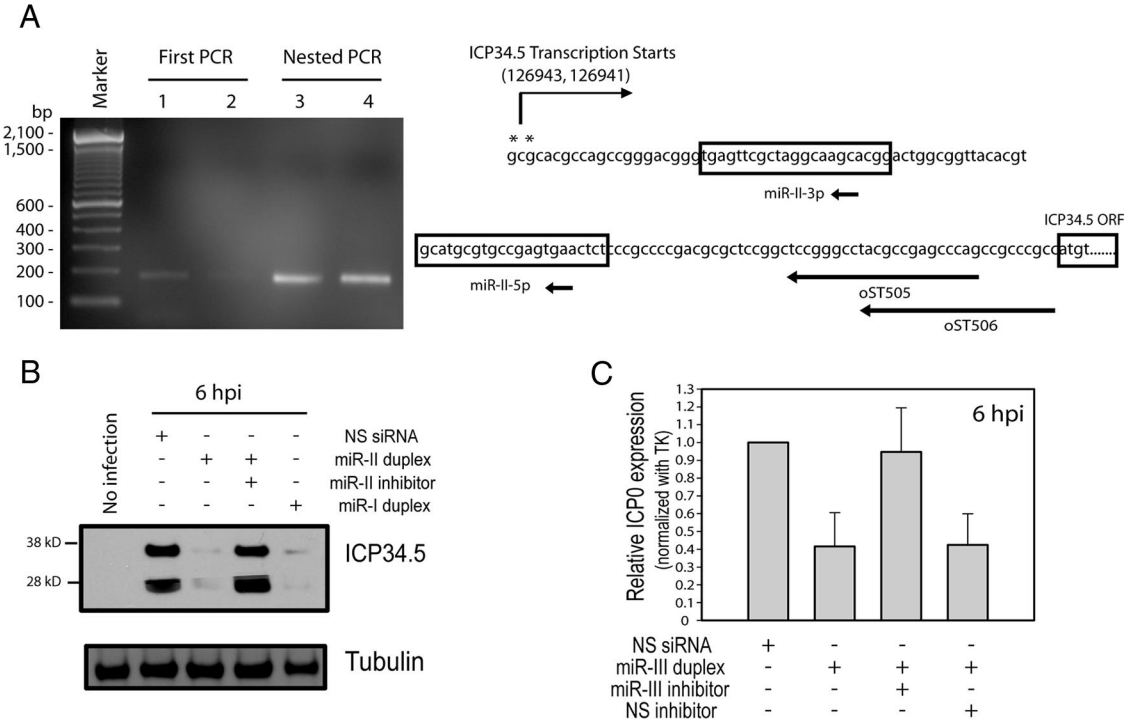

FIG. 4. miR-II and miR-III inhibit ICP34.5 and ICP0 expression, respectively. (A) Mapping of the transcription initiation site of the HSV-2 ICP34.5 gene. Ten micrograms of total RNA was obtained at 16 h after inoculation of Vero cells with HSV-2 strain 333-infected Vero cells and used to analyze the 5′ transcription initiation site of the ICP34.5 gene. Two partially overlapping primers, oST505 and oST506, were used as gene-specific primers in 5′ RACE. cDNA was amplified with a 5′ RACE outer primer and oST505 (lane 1) or oST506 (lane 2). The first PCR products were further amplified with a nested 5′ RACE inner primer and oST505 (lane 3) or oST506 (lane 4). The nested PCR product from primers oST506 and the 5′ RACE inner primer was cloned and sequenced. The transcription initiation sites of the ICP34.5 gene map to nt 126943 (16 of 22 clones sequenced) and 126941 (6 of 22 clones). Asterisks indicate the transcription start sites. The locations of the ICP34.5 gene open reading frame (ORF), miR-II, and the primers are also labeled. (B) miR-II specifically silences ICP34.5 expression. U2OS cells were transfected with 20 nM miR-II duplex with or without 40 nM miR-II-5p-specific inhibitor 16 h before infection with HSV-2 strain 333 at a MOI of 2. Twenty nanomolar nonspecific (NS) siRNA (Ambion) and the miR-I duplex were also transfected and used as negative and positive controls, respectively. Total proteins were extracted at 6 hpi and separated on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel before transfer to a membrane and incubation with an HSV-2 ICP34.5-specific antibody. The same membrane was stripped and incubated with an anti-β-tubulin antibody as a loading control. (C) miR-III specifically reduces ICP0 mRNA levels. U2OS cells were transfected with 20 nM miR-III duplex with or without 40 nM miR-III-specific inhibitor or NS miRNA inhibitor 16 h before infection with HSV-2 strain 333 at a MOI of 2. Twenty nanomolar NS siRNA (Dharmacon) was also transfected and used as a negative control. Total RNAs were prepared at 6 hpi and ICP0 and TK levels were analyzed by real-time PCR. Expression of ICP0 was normalized to TK and is presented as relative ICP0 expression. The panel summarizes five independent experiments.

HincII-transfected cells, because pPstI-HincII does not contain the miR-III region.

The mutant virus R4530 has an HSV-1 ORF-O, P ICP4-binding site mutation and expresses increased ORF-O, P but reduced ICP34.5 mRNA in infected cells (26). We used Northern hybridization to test whether destruction of the ORF-O, P ICP4-binding site affects LAT-encoded miRNA expression. R4530 expresses more miR-LAT-ICP34.5 (miR-H4-3p) than R4531 (its rescuant virus) in both Vero and SK-N-SH cells (Fig. 3C), suggesting that ICP4 is involved in the regulation of miR-LAT-ICP34.5 (miR-H4-3p) expression in infected cells.

Determination of the transcription initiation site for the HSV-2 ICP34.5 gene. Unlike miR-I, miR-II-5p and miR-II3p do not map to the antisense strand of the coding region of the ICP34.5 gene but to the antisense strand of the potential 5′ UTR of the ICP34.5 gene. Since there is no canonical TATA box for the HSV-2 ICP34.5 gene, it is impossible to predict the ICP34.5 transcription initiation site based on the sequence. To investigate whether or not miR-II is antisense to the 5′ UTR of the ICP34.5 transcript, we used a 5′ RACE assay to map the 5′ transcription initiation site for ICP34.5 in HSV-2-infected Vero cells. Two partially overlapped primers (oST505 and oST506) were used as gene-specific primers (Fig. 4A). The transcription initiation sites of ICP34.5 were mapped to nt 126943 (16 of 22 clones sequenced) and nt 126941 (6 of 22 clones). Both miR-II-5p and miR-II-3p thus map to the 5′ UTR of the ICP34.5 transcript in an antisense direction (Fig. 4A), as does the homologous miR-LAT-ICP34.5 (miR-H4-3p) for HSV-1 (8, 48), suggesting that ICP34.5 could be the target of both miR-I and miR-II.

HSV-2 LAT-encoded miRNAs specifically reduce ICP34.5 and ICP0 expression. We previously showed that miR-I is able to reduce ICP34.5 expression by functioning as a small interfering RNA (siRNA) (40). Since miR-II maps to the antisense strand of the 5′ UTR of the ICP34.5 gene, it seems likely that both miR-I and miR-II share the same target, ICP34.5. To test whether miR-II can reduce ICP34.5 expression, we transfected U2OS cells with the miR-II duplex with or without a miR-II-

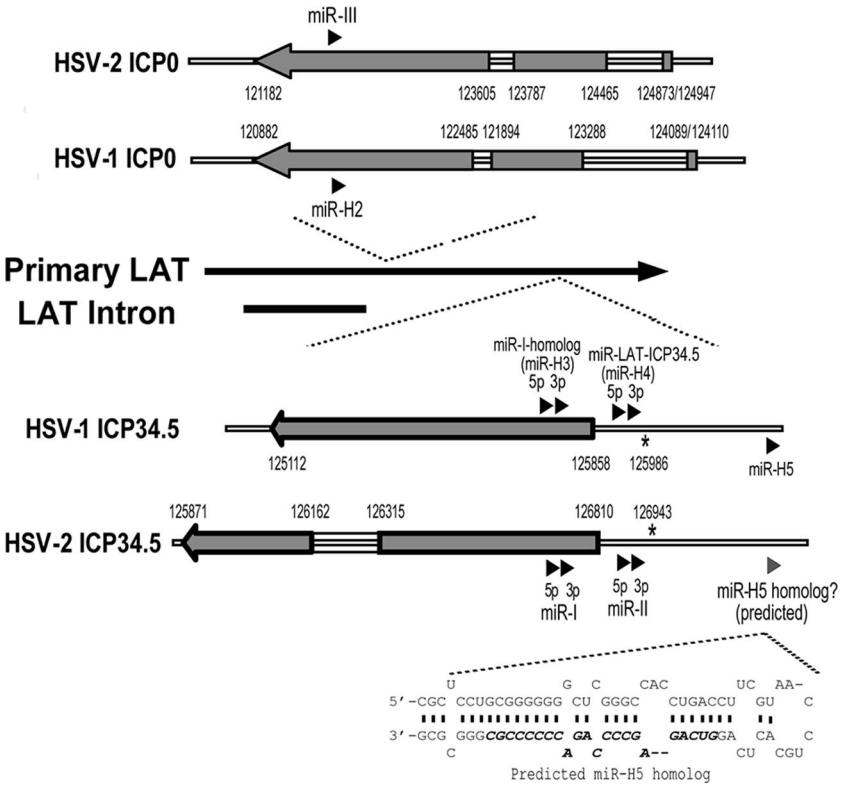

FIG. 5. Schematic diagram of the HSV-1 and HSV-2 LAT-encoded miRNAs. miR-I-homolog-3p is the same as a recently identified miRNA, miR-H3; miR-LAT-ICP34.5 is the same as miR-H4 (40, 48). miR-I is 77% homologous to miR-I-homolog (miR-H3), while miR-II-3p is 76% homologous to miR-LAT-ICP34.5 (miR-H4-3p). miR-III and miR-H2 are similar in genome location but have no homology. The hypothesized HSV-2 miR-H5 homolog (in boldface italics) is predicted based on homology search and maps to LAT exon 2 (nt 127634 to 127655). The miR-H5 homolog and its precursor are under the detection limit of Northern hybridization in infected- and transfected-cell cultures (data not shown). Asterisks indicate the transcription start sites.

5p-specific inhibitor 16 h before infection with HSV-2. Similar to miR-I, miR-II efficiently reduced ICP34.5 protein expression (Fig. 4B). A miR-II-5p-specific miRNA inhibitor relieved the inhibition of ICP34.5 expression by miR-II, indicating that, similar to miR-I, miR-II can downregulate ICP34.5 expression specifically.

To test whether miR-III can reduce ICP0 expression, we transfected U2OS cells with a miR-III duplex with or without a miR-III-specific inhibitor 16 h before infection with HSV-2 strain 333. Since an anti-HSV-2 ICP0 antibody is not available, we used real-time PCR to detect the expression of ICP0 mRNA from these infected cells. In five independent experiments, the miR-III duplex reduced ICP0 expression by approximately 60% with no effect on TK expression (Fig. 4C). A specific miR-III inhibitor, but not the nonspecific miRNA inhibitor, relieved the inhibition of ICP0 expression by the miR-III duplex (Fig. 4C).

Comparison of HSV-1 and HSV-2 LAT-encoded miRNAs. Because HSV-1 and HSV-2 are closely related, identification of these less-abundant HSV-2 LAT-encoded miRNAs provides a unique opportunity to compare the evolutionary paths of virally encoded miRNAs in these two closely related human herpesviruses (Fig. 5). The three identified HSV-2 LAT-encoded miRNAs match three out of four HSV-1 miRNAs based on their genome locations. There is minimal sequence similarity between miR-III and HSV-1 miR-H2. Although miR-I shares approximately 77% homology with HSV-1 miR-I-homolog-3p (HSV-1 miR-H3) and miR-II-3p shares 76% homology with miR-LAT-ICP34.5 (HSV-1 miR-H4-3p), it is difficult to find similarities among the first 8 nt, which are most important for miRNA functions (1), implying that the potential nonviral targets of these homologous miRNAs (if any) are dramatically different from one another. Therefore, it seems likely that the major evolutionary pressure on the HSV-1 and HSV-2 LAT-encoded miRNAs is to maintain the RNA secondary structures to allow processing into miRNAs by Drosha and Dicer but not to keep the miRNA sequences unchanged during the approximately nine million years of evolution since these two closely related viruses began to diverge (17). Thus, these LAT-encoded miRNAs seem more likely to have coevolved with their viral targets rather than potential cellular targets.

A predicted HSV-1 miR-H5 homolog in the HSV-2 genome is not detectable in infected and transfected cells. Since HSV LAT-encoded miRNAs are most conserved in location, we predicted that HSV-2 may also encode an HSV-1 miR-H5 homolog miRNA in a similar genome location, although HTP sequencing did not reveal such a miRNA-like sequence. Through a homology search with the Vector NTI program (Invitrogen, CA), we identified an HSV-2 sequence that is 76% homologous to HSV-1 miR-H5 in a location similar to that of HSV-1 miR-H5. This miR-H5 homolog can potentially fold into a stem-loop structure with adjacent upstream sequences

(Fig. 5). However, the mature forms of any HSV-2 miR-H5 homolog and its precursor are below the detection level of Northern hybridization in Vero cells infected with HSV-2 strains 333 and HG52 or HEK 293 cells transfected with pCMV-SSK (data not shown), suggesting that evolutionary drift of miR-H5 homolog sequences is significant enough to affect its processing by the miRNA machinery. Together with the absence of an overlapping viral target and differences in 5′ sequence that imply no common cellular target, these data also suggest that HSV-1 miR-H5 may not contribute to the function of LAT as much as other LAT-encoded miRNAs.

DISCUSSION

We previously reported on an HSV-2 LAT-encoded miRNA, miR-I, which is highly expressed during latent and acute infection and is able to downregulate ICP34.5 expression (40). In the present study, we used 454 HTP sequencing technology to identify two additional relatively less-abundant HSV-2 viral miRNAs (miR-II and miR-III) in LAT exon 2. miR-II maps to the antisense strand of the 5′ UTR of the HSV-2 ICP34.5 gene, while miR-III maps to the antisense strand of the coding region of ICP0 exon 3. These novel miRNAs were detected by Northern hybridization in both infected- and transfected-cell cultures. We further demonstrated that, similar to miR-I, miR-II is able to silence the expression of ICP34.5, a key viral neurovirulence factor, and that miR-III is able to silence the expression of ICP0, a key viral transactivator.

Transcription of the LAT-encoded miRNAs seems to be tightly regulated. During latency, the LAT promoter is highly active in terminally differentiated sensory neuronal cells. We previously showed that miR-I is highly expressed by the LAT promoter during latency in vivo in guinea pigs and humans (40). It is likely that all LAT-encoded miRNAs, including miR-II and -III, are also actively expressed during latency in vivo, although miR-I seems to be dominant. Promoters other than the LAT promoter, including the putative HSV-2 ORF-O, P promoter (a pre-α promoter in HSV-1 [3]), also likely contribute to miR-I and miR-II expression (Fig. 2), which introduces another layer of transcriptional control. This implies that the regulation of miR-I and miR-II may be more complicated than that of miR-III. Interestingly, ICP4, the major viral transactivator, inhibits both the LAT and the ORF-O, P promoters through sequence-specific binding at transcription start sites, thus suppressing expression of LAT-encoded miRNAs (Fig. 3). ICP4 is required for most post-α gene expression and viral replication (9, 12, 24). It seems likely that ICP4 expression inhibits LAT-encoded miRNAs to create an environment more favorable for viral reactivation. The unique transcriptional regulation of LAT-encoded miRNAs may enable LAT-encoded miRNAs to function as a molecular switch between HSV latency and reactivation in infected ganglia.

LAT-encoded miRNAs from HSV-2 and HSV-1, two closely related human herpesviruses, are more conserved in location than in sequence (Fig. 5). Three out of four miRNAs identified in the HSV-1 LAT have a counterpart in a similar location in HSV-2. Although there is some homology between the miRNAs encoded by HSV-1 and HSV-2, the first 2 to 8 5′ nucleotides, which determine the miRNA targets, differ dramatically between the corresponding HSV-1 and HSV-2 miRNAs; thus, it seems very unlikely that these miRNAs have similar cellular targets, suggesting that viral mRNAs are more likely to be targets of the LAT-encoded miRNAs. One difference between these two viruses is in the observed expression levels of the viral miRNAs. In HSV-2, miR-I is the most abundant miRNA. In HSV-1, miR-H2, the HSV-2 miR-III homolog (whose target is ICP0), is the most abundant miRNA (48). The expression level of the miRNAs likely affects their function, which may help to explain the different behaviors during latency of these two closely related viruses (15, 29, 50).

We previously demonstrated that miR-I inhibits expression of ICP34.5, the key viral neurovirulence factor. In this study, we further demonstrate that miR-II can also silence ICP34.5 expression in a sequence-specific manner by targeting the 5′ UTR of the gene. ICP34.5 deletion mutants show significantly decreased replication in human brain (31, 34, 36). Although the ICP34.5 gene is characterized as a γ1 late gene (8), ICP34.5 is detectable as early as 2 hpi in infected-cell cultures (23). The C terminus of ICP34.5 is highly homologous to the corresponding domain of a conserved mammalian protein known as GADD34 and is responsible for its function as a protein kinase R inhibitor (20, 21). It is generally believed that ICP34.5 is a late acquisition by HSV. It is likely that, when the conserved and functional domain of ICP34.5 was acquired, negative regulating elements, including two conserved miRNAs, were also acquired by the sequence encoding the N terminus and by the 5′ UTR of its gene. Two out of three HSV LAT-encoded miRNAs that are conserved in location target ICP34.5, further strengthening our hypothesis that control of ICP34.5 expression in individual infected neurons by these LAT-encoded miRNAs affects the outcome (i.e., productive infection versus latency) of infection in those neurons, leading either to spread to other neurons or to direct establishment of latency (40).

The LAT was originally hypothesized to control lytic gene expression via an antisense interaction with ICP0 (16, 39). The ICP0 gene is an IE viral gene and plays an important role in HSV reactivation (4, 5, 18, 19). Although recent studies indicate that ICP0 null mutants can be induced to reactivate, these mutants were not shown to reactivate in vivo and the efficiency of latency establishment for these mutants was reduced (33, 43). We show in this report that miR-III is able to reduce ICP0 mRNA expression, although not as efficiently as miR-I and miR-II reduce ICP34.5 expression. Interestingly, HSV-1 miR-H2, which shares a similar viral genome location with miR-III, also inhibits ICP0 expression (48). These data suggest that control of HSV ICP0 expression could be an important step in establishment and maintenance of latency.

It has been suggested that the effect on LAT during latency is quantitative rather than absolute. The cumulative function of these LAT-encoded miRNAs, which appear to modulate ICP0 and ICP34.5 expression at late times after infection, may contribute to these phenomena. It is still not known to what extent LAT-encoded miRNAs contribute to latency and reactivation in vivo. While miRNA inhibitors reversed the ability of exogenously delivered miRNAs to silence ICP0 and ICP34.5, the inhibitors had no obvious effect on viral replication or gene expression in infected U2OS cells (data not shown). This may be due to the limited sensitivity of our cell culture systems to any such effect. Further studies of LAT-encoded miRNA-neg-

ative mutant viruses are thus needed to delineate the function of these miRNAs in vivo.

ACKNOWLEDGMENTS

We thank all members of the Krause lab. We also thank Bernard Roizman for providing the ICP4-binding site mutant viruses (R7530 and its rescuant virus, R7531) and Jeffrey Cohen and Kening Wang for providing the HSV-2 ICP4 expression plasmid.

This study was supported by the intramural research programs of the Center for Biologics Evaluation and Research.

REFERENCES

1. **Bartel, D. P.** 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell **116:**281–297.
2. **Bartel, D. P., and C. Z. Chen.** 2004. Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. Nat. Rev. Genet. **5:**396–400.
3. **Bohenzky, R. A., A. G. Papavassiliou, I. H. Gelman, and S. Silverstein.** 1993. Identification of a promoter mapping within the reiterated sequences that flank the herpes simplex virus type 1 UL region. J. Virol. **67:**632–642.
4. **Cai, W., T. L. Astor, L. M. Liptak, C. Cho, D. M. Coen, and P. A. Schaffer.** 1993. The herpes simplex virus type 1 regulatory protein ICP0 enhances virus replication during acute infection and reactivation from latency. J. Virol. **67:**7501–7512.
5. **Cai, W. Z., and P. A. Schaffer.** 1989. Herpes simplex virus type 1 ICP0 plays a critical role in the de novo synthesis of infectious virus following transfection of viral DNA. J. Virol. **63:**4579–4589.
6. **Chou, J., E. R. Kern, R. J. Whitley, and B. Roizman.** 1990. Mapping of herpes simplex virus-1 neurovirulence to gamma 134.5, a gene nonessential for growth in culture. Science **250:**1262–1266.
7. **Chou, J., and B. Roizman.** 1992. The gamma 1(34.5) gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programmed cell death in neuronal cells. Proc. Natl. Acad. Sci. USA **89:**3266–3270.
8. **Chou, J., and B. Roizman.** 1986. The terminal a sequence of the herpes simplex virus genome contains the promoter of a gene located in the repeat sequences of the L component. J. Virol. **57:**629–637.
9. **Clements, J. B., R. J. Watson, and N. M. Wilkie.** 1977. Temporal regulation of herpes simplex virus type 1 transcription: location of transcripts on the viral genome. Cell **12:**275–285.
10. **Cui, C., A. Griffiths, G. Li, L. M. Silva, M. F. Kramer, T. Gaasterland, X. J. Wang, and D. M. Coen.** 2006. Prediction and identification of herpes simplex virus 1-encoded microRNAs. J. Virol. **80:**5499–5508.
11. **Cullen, B. R.** 2006. Viruses and microRNAs. Nat. Genet. **38**(Suppl.)**:**S25–S30.
12. **Dixon, R. A., and P. A. Schaffer.** 1980. Fine-structure mapping and functional analysis of temperature-sensitive mutants in the gene encoding the herpes simplex virus type 1 immediate early protein VP175. J. Virol. **36:**189–203.
13. **Farrell, M. J., A. T. Dobson, and L. T. Feldman.** 1991. Herpes simplex virus latency-associated transcript is a stable intron. Proc. Natl. Acad. Sci. USA **88:**790–794.
14. **Farrell, M. J., T. P. Margolis, W. A. Gomes, and L. T. Feldman.** 1994. Effect of the transcription start region of the herpes simplex virus type 1 latency-associated transcript promoter on expression of productively infected neurons in vivo. J. Virol. **68:**5337–5343.
15. **Feldman, L. T., A. R. Ellison, C. C. Voytek, L. Yang, P. Krause, and T. P. Margolis.** 2002. Spontaneous molecular reactivation of herpes simplex virus type 1 latency in mice. Proc. Natl. Acad. Sci. USA **99:**978–983.
16. **Garber, D. A., P. A. Schaffer, and D. M. Knipe.** 1997. A LAT-associated function reduces productive-cycle gene expression during acute infection of murine sensory neurons with herpes simplex virus type 1. J. Virol. **71:**5885–5893.
17. **Gentry, G. A., M. Lowe, G. Alford, and R. Nevins.** 1988. Sequence analyses of herpesviral enzymes suggest an ancient origin for human sexual behavior. Proc. Natl. Acad. Sci. USA **85:**2658–2661.
18. **Hagglund, R., and B. Roizman.** 2004. Role of ICP0 in the strategy of conquest of the host cell by herpes simplex virus 1. J. Virol. **78:**2169–2178.
19. **Halford, W. P., and P. A. Schaffer.** 2001. ICP0 is required for efficient reactivation of herpes simplex virus type 1 from neuronal latency. J. Virol. **75:**3240–3249.
20. **He, B., M. Gross, and B. Roizman.** 1998. The γ134.5 protein of herpes simplex virus 1 has the structural and functional attributes of a protein phosphatase 1 regulatory subunit and is present in a high molecular weight complex with the enzyme in infected cells. J. Biol. Chem. **273:**20737–20743.
21. **He, B., M. Gross, and B. Roizman.** 1997. The $\gamma_1$34.5 protein of herpes simplex virus 1 complexes with protein phosphatase 1α to dephosphorylate the alpha subunit of the eukaryotic translation initiation factor 2 and preclude the shutoff of protein synthesis by double-stranded RNA-activated protein kinase. Proc. Natl. Acad. Sci. USA **94:**843–848.
22. **He, L., and G. J. Hannon.** 2004. MicroRNAs: small RNAs with a big role in gene regulation. Nat. Rev. Genet. **5:**522–531.
23. **Holman, H. A., and A. R. MacLean.** 2008. Neurovirulent factor ICP34.5 uniquely expressed in the herpes simplex virus type 1 Delta gamma 1 34.5 mutant 1716. J. Neurovirol. **14:**28–40.
24. **Knipe, D. M., W. T. Ruyechan, B. Roizman, and I. W. Halliburton.** 1978. Molecular genetics of herpes simplex virus: demonstration of regions of obligatory and nonobligatory identity within diploid regions of the genome by sequence replacement and insertion. Proc. Natl. Acad. Sci. USA **75:**3896–3900.
25. **Krause, P. R., L. R. Stanberry, N. Bourne, B. Connelly, J. F. Kurawadwala, A. Patel, and S. E. Straus.** 1995. Expression of the herpes simplex virus type 2 latency-associated transcript enhances spontaneous reactivation of genital herpes in latently infected guinea pigs. J. Exp. Med. **181:**297–306.
26. **Lagunoff, M., G. Randall, and B. Roizman.** 1996. Phenotypic properties of herpes simplex virus 1 containing a derepressed open reading frame P gene. J. Virol. **70:**1810–1817.
27. **Lee, L. Y., and P. A. Schaffer.** 1998. A virus with a mutation in the ICP4-binding site in the L/ST promoter of herpes simplex virus type 1, but not a virus with a mutation in open reading frame P, exhibits cell-type-specific expression of $\gamma_1$34.5 transcripts and latency-associated transcripts. J. Virol. **72:**4250–4264.
28. **Leib, D. A., C. L. Bogard, M. Kosz-Vnenchak, K. A. Hicks, D. M. Coen, D. M. Knipe, and P. A. Schaffer.** 1989. A deletion mutant of the latency-associated transcript of herpes simplex virus type 1 reactivates from the latent state with reduced frequency. J. Virol. **63:**2893–2900.
29. **Margolis, T. P., Y. Imai, L. Yang, V. Vallas, and P. R. Krause.** 2007. Herpes simplex virus type 2 (HSV-2) establishes latent infection in a different population of ganglionic neurons than HSV-1: role of latency-associated transcripts. J. Virol. **81:**1872–1878.
30. **Margulies, M., M. Egholm, W. E. Altman, S. Attiya, J. S. Bader, L. A. Bemben, J. Berka, M. S. Braverman, Y. J. Chen, Z. Chen, S. B. Dewell, L. Du, J. M. Fierro, X. V. Gomes, B. C. Godwin, W. He, S. Helgesen, C. H. Ho, G. P. Irzyk, S. C. Jando, M. L. Alenquer, T. P. Jarvie, K. B. Jirage, J. B. Kim, J. R. Knight, J. R. Lanza, J. H. Leamon, S. M. Lefkowitz, M. Lei, J. Li, K. L. Lohman, H. Lu, V. B. Makhijani, K. E. McDade, M. P. McKenna, E. W. Myers, E. Nickerson, J. R. Nobile, R. Plant, B. P. Puc, M. T. Ronan, G. T. Roth, G. J. Sarkis, J. F. Simons, J. W. Simpson, M. Srinivasan, K. R. Tartaro, A. Tomasz, K. A. Vogt, G. A. Volkmer, S. H. Wang, Y. Wang, M. P. Weiner, P. Yu, R. F. Begley, and J. M. Rothberg.** 2005. Genome sequencing in microfabricated high-density picolitre reactors. Nature **437:**376–380.
31. **Markert, J. M., M. D. Medlock, S. D. Rabkin, G. Y. Gillespie, T. Todo, W. D. Hunter, C. A. Palmer, F. Feigenbaum, C. Tornatore, F. Tufaro, and R. L. Martuza.** 2000. Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: results of a phase I trial. Gene Ther. **7:**867–874.
32. **Perng, G. C., C. Jones, J. Ciacci-Zanella, M. Stone, G. Henderson, A. Yukht, S. M. Slanina, F. M. Hofman, H. Ghiasi, A. B. Nesburn, and S. L. Wechsler.** 2000. Virus-induced neuronal apoptosis blocked by the herpes simplex virus latency-associated transcript. Science **287:**1500–1503.
33. **Preston, C. M.** 2007. Reactivation of expression from quiescent herpes simplex virus type 1 genomes in the absence of immediate-early protein ICP0. J. Virol. **81:**11781–11789.
34. **Rampling, R., G. Cruickshank, V. Papanastassiou, J. Nicoll, D. Hadley, D. Brennan, R. Petty, A. MacLean, J. Harland, E. McKie, R. Mabbs, and M. Brown.** 2000. Toxicity evaluation of replication-competent herpes simplex virus (ICP 34.5 null mutant 1716) in patients with recurrent malignant glioma. Gene Ther. **7:**859–866.
35. **Sawtell, N. M., and R. L. Thompson.** 1992. Herpes simplex virus type 1 latency-associated transcription unit promotes anatomical site-dependent establishment and reactivation from latency. J. Virol. **66:**2157–2169.
36. **Spivack, J. G., M. U. Fareed, T. Valyi-Nagy, T. C. Nash, J. S. O'Keefe, R. M. Gesser, E. A. McKie, A. R. MacLean, N. W. Fraser, and S. M. Brown.** 1995. Replication, establishment of latent infection, expression of the latency-associated transcripts and explant reactivation of herpes simplex virus type 1 gamma 34.5 mutants in a mouse eye model. J. Gen. Virol. **76:**321–332.
37. **Steiner, I., N. Mador, I. Reibstein, J. G. Spivack, and N. W. Fraser.** 1994. Herpes simplex virus type 1 gene expression and reactivation of latent infection in the central nervous system. Neuropathol. Appl. Neurobiol. **20:**253–260.
38. **Steiner, I., J. G. Spivack, R. P. Lirette, S. M. Brown, A. R. MacLean, J. H. Subak-Sharpe, and N. W. Fraser.** 1989. Herpes simplex virus type 1 latency-associated transcripts are evidently not essential for latent infection. EMBO J. **8:**505–511.
39. **Stevens, J. G., E. K. Wagner, G. B. Devi-Rao, M. L. Cook, and L. T. Feldman.** 1987. RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. Science **235:**1056–1059.
40. **Tang, S., A. S. Bertke, A. Patel, K. Wang, J. I. Cohen, and P. R. Krause.** 2008. An acutely and latently expressed herpes simplex virus 2 viral microRNA inhibits expression of ICP34.5, a viral neurovirulence factor. Proc. Natl. Acad. Sci. USA **105:**10931–10936.

41. **Tang, S., M. Tao, J. P. McCoy, Jr., and Z. M. Zheng.** 2006. Short-term induction and long-term suppression of HPV16 oncogene silencing by RNA interference in cervical cancer cells. Oncogene **25:**2094–2104.
42. **Tang, S., K. Yamanegi, and Z. M. Zheng.** 2004. Requirement of a 12-base-pair TATT-containing sequence and viral lytic DNA replication in activation of the Kaposi's sarcoma-associated herpesvirus K8.1 late promoter. J. Virol. **78:**2609–2614.
43. **Thompson, R. L., and N. M. Sawtell.** 2006. Evidence that the herpes simplex virus type 1 ICP0 protein does not initiate reactivation from latency in vivo. J. Virol. **80:**10919–10930.
44. **Thompson, R. L., and N. M. Sawtell.** 2001. Herpes simplex virus type 1 latency-associated transcript gene promotes neuronal survival. J. Virol. **75:** 6660–6675.
45. **Thompson, R. L., and N. M. Sawtell.** 1997. The herpes simplex virus type 1 latency-associated transcript gene regulates the establishment of latency. J. Virol. **71:**5432–5440.
46. **Thompson, R. L., and N. M. Sawtell.** 2000. HSV latency-associated transcript and neuronal apoptosis. Science **289:**1651.
47. **Trousdale, M. D., I. Steiner, J. G. Spivack, S. L. Deshmane, S. M. Brown, A. R. MacLean, J. H. Subak-Sharpe, and N. W. Fraser.** 1991. In vivo and in vitro reactivation impairment of a herpes simplex virus type 1 latency-associated transcript variant in a rabbit eye model. J. Virol. **65:**6989–6993.
48. **Umbach, J. L., M. F. Kramer, I. Jurak, H. W. Karnowski, D. M. Coen, and B. R. Cullen.** 2008. MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. Nature **454:**780–783.
49. **Whitley, R. J., E. R. Kern, S. Chatterjee, J. Chou, and B. Roizman.** 1993. Replication, establishment of latency, and induced reactivation of herpes simplex virus gamma 1 34.5 deletion mutants in rodent models. J. Clin. Investig. **91:**2837–2843.
50. **Yoshikawa, T., J. M. Hill, L. R. Stanberry, N. Bourne, J. F. Kurawadwala, and P. R. Krause.** 1996. The characteristic site-specific reactivation phenotypes of HSV-1 and HSV-2 depend upon the latency-associated transcript region. J. Exp. Med. **184:**659–664.