Herpes Simplex Virus 2 Latency-Associated Transcript (LAT) Region Mutations Do Not Identify a Role for LAT-Associated MicroRNAs in Viral Reactivation in Guinea Pig Genital Models

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ABSTRACT Despite the long-standing observation that herpes simplex virus (HSV) latency-associated transcript (LAT) promoter deletion viruses show impaired recurrence phenotypes in relevant animal models, the mechanism by which these sequences exert this phenotypic effect is unknown. We constructed and evaluated four mutant HSV-2 isolates with targeted mutations in the LAT promoter and LAT-associated microRNAs (miRNAs) affecting (i) the LAT TATA box; (ii) the LAT ICP4-binding site; (iii) miRNA I (miR-I) and miR-II (miR-I/II), which both target ICP34.5; and (iv) miR-III, which targets ICP0. While the LAT TATA box mutant caused milder acute infections than wild-type (WT) virus, there was no difference in the recurrence phenotype between these viruses. LAT and miRNA expression during latency was not impaired by this mutation, suggesting that other promoter elements may be more important for latent HSV-2 LAT expression. Mutation of the LAT ICP4-binding site also did not cause an *in vivo* phenotypic difference between mutant and WT viruses. Acute infection and reactivation from latency of the miR-I/II mutant were similar to those of its rescuer, although the acute infection was slightly reduced in severity relative to that caused by the wild-type virus. The miR-III mutant also exhibited WT phenotypes in acute and recurrent phases of infection. While they do not rule out an effect of these elements in human latency or reactivation, these findings do not identify a specific role for LAT or LAT-associated miRNAs in the HSV-2 LAT promoter deletion phenotype in guinea pigs. Thus, other sequences in this region may play a more important role in the long-studied LAT-associated phenotype in animals.

IMPORTANCE While it has been known for several decades that specific HSV-1 and HSV-2 sequences near the LAT promoter are required for efficient viral reactivation in animal models, the mechanism is still not known. We constructed four mutant viruses with the goal of identifying critical sequence elements and of specifically testing the hypothesis that microRNAs that are expressed during latency play a role. Determination that specific LAT promoter sequences and miRNA sequences do not influence viral reactivation of HSV-2 helps to narrow down the search for the mechanism by which the virus controls its latency and recurrence phenotype.

KEYWORDS latency

Herpes simplex virus 1 (HSV-1) and HSV-2 are closely related human herpesviruses. HSV-1 typically infects the facial region and preferentially reactivates from latency in the sensory trigeminal ganglia, while HSV-2 typically infects the genital region and reactivates from latency in the sensory sacral dorsal root ganglia (DRGs). Both viruses can also infect and establish latency in autonomic neurons. Periodically, in response to various stimuli, either virus can reactivate to cause recurrences in the area served by these sensory neurons (1).

The most abundant viral transcript during latency of both HSV-1 and HSV-2 is the

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noncoding latency-associated transcript (LAT), which is transcribed from within the long repeats of the viral genome (2). 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. The stable 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 spliced from the less stable ~8.5-kb primary LAT, which starts 25 nucleotides downstream of the LAT TATA box (3) and overlaps transcripts of the E3 ubiquitin ligase ICP0 and of the α subunit of eukaryotic initiation factor 2 dephosphorylase and neurovirulence factor ICP34.5 in an antisense direction (4–8). The primary LAT is a pri-microRNA (miRNA) transcript, yielding miRNAs that inhibit expression of ICP34.5 and ICP0 (9). Deletion of the LAT promoter in both HSV-1 and HSV-2 reduces the efficiency of reactivation (10–17), and substitution of HSV-1 LAT for native HSV-2 LAT sequences confers an HSV-1 reactivation phenotype (18). This phenotype appears not to be due to differences in the HSV-1 and HSV-2 LAT promoters but instead maps to sequences downstream of the promoter (19), which contain enhancer sequences (20) and an ICP4-binding site, through which ICP4 inhibits LAT promoter activity *in vitro* (3). Various mutations in the HSV-1 LAT, including intron deletion (21) and destabilization of the intron by mutation of its splice branch point (22), have no effect on the reactivation phenotype. Mutation of HSV-1 miRNA H2 (miR-H2; corresponding to miR-III in HSV-2), which inhibits ICP0 expression, was variably reported to increase (23) or have no impact on (24) viral reactivation. Deleting HSV-1 miRNAs miR-H3 and miR-H4, which correspond to miR-I and miR-II in HSV-2, respectively, and which target ICP34.5, increased viral replication in neuronal cell culture (25). While it is hypothesized that the control of ICP34.5 expression in individual infected neurons by these LAT-encoded miRNAs affects the outcome of infection (i.e., productive infection versus latency), leading either to spread to other neurons or to direct establishment of latency (9, 26), this has not been demonstrated. To date, no experiments have evaluated the impact of miRNA expression on HSV-2 acute and recurrent infection *in vivo*.

In the present study, to further understand the role in viral reactivation of HSV-2 LAT region sequences, including miRNAs, we constructed and characterized the infection and recurrence phenotypes of mutant viruses with focused deletions in the miRNAs and in the LAT promoter. While a NotI-NotI mutant HSV-2 that does not contain the LAT promoter or ICP4-binding sites and which does not express the LAT intron or LAT-associated miRNAs during latency displayed impaired reactivation (12), we reasoned that more targeted LAT promoter mutants, if they influenced miRNA expression, might help in evaluating the combined effect of changing expression of all of the LAT-associated miRNAs. The specific mutations targeted the HSV-2 LAT TATA box, the ICP4-binding site, miR-I and miR-II (miR-I/II), and miR-III. When recurrence phenotypes differed between wild-type (WT) and mutant viruses, we also constructed and tested a rescuant to verify that the phenotype was due to sequences within the mutated region. Tissues also were evaluated for viral DNA and ICP0 and LAT transcripts during latent infection.

RESULTS

Construction of HSV-2 LAT TATA box, ICP4-binding site, miR-I/II, and miR-III mutants. Figure 1 shows the relevant genomic regions, restriction endonuclease cleavage sites, plasmids, and PCR fragments. The construction of the HSV-2 LAT TATA box, ICP4-binding site, miR-I/II, and miR-III mutants and rescuants of the miR-I/II or miR-III mutants was verified by sequencing of the mutated regions from the constructed viruses (Table 1). The impact of the mutations of miR-I, miR-II, and miR-III on pre-miRNA expression was also studied by Northern hybridization of infected cell cultures, indicating that each of these mutations disrupted pre-miRNA expression (Fig. 2A and B). These experiments also showed elimination of miR-III expression in the miR-III mutant (Fig. 2B). In wild-type virus-infected cells, miR-I expression was not detected because the probe does not overlap miR-I, and miR-II likely was not detected because the quantity of miR-II expressed was too low for detection in this experiment.

FIG 1 Schematic diagram of HSV-2 LAT region with relevant plasmid and PCR fragments. The HSV genome consists of unique long and short regions (U_L and U_S, respectively) flanked by long and short internal repeats (IR_L and IR_S, respectively) and long and short terminal repeats (TR_L and TR_S, respectively). The primary LAT is transcribed from the repeat regions in the direction antisense to ICP0 and ICP34.5 (transcripts are shown here as lines, with arrows indicating the direction of transcription). The primary LAT is spliced into a single stable LAT intron in HSV-2. Boxes on the ICP0 and ICP34.5 transcripts denote introns that are not readily detectable after splicing. The diagrammed plasmids and PCR fragments were used to produce the corresponding mutant and rescuant viruses.

Mutation of the LAT TATA box decreased expression of LAT *in vitro* in transfected cells but not in infected cells. To determine whether the LAT TATA box mutation alters LAT expression, we performed Northern hybridization using RNA extracted from human embryonic kidney 293 (HEK 293) cells after transfection of a plasmid with a LAT TATA substitution (the pSSB-TATA mutant). The 2.2-kb HSV-2 LAT was not detected in cells transfected with the LAT promoter deletion construct (pSSB-Not⁻) or in untransfected cells but was readily detected in wild-type plasmid (pSSB)-transfected cells. Although HSV-2 LAT was also detected in pSSB-TATA mutant-transfected cells, the quantity of LAT was reduced relative to that of pSSB (Fig. 3A). However, *in vitro* growth in Vero cells of the HSV-2 LAT TATA mutant was indistinguishable from that of wild-type HSV-2 (Fig. 3B).

The HSV-2 LAT TATA box does not influence the viral recurrence phenotype in guinea pigs. To determine if the HSV-2 LAT TATA box is required for the efficient reactivation of HSV-2 in guinea pigs, female guinea pigs were infected with the HSV-2 WT or the HSV-2 LAT TATA box mutants. The mean lesion score during the acute phase of infection through day 14 postinoculation (p.i.) in guinea pigs infected with the HSV-2

TABLE 1 Comparison of sequences in the wild-type (HSV-2 333) and mutant viruses used in this study

Region	Positions ^a	Virus	Sequence ^b (5' to 3')
TATA box	119507–119512	HSV-2 333	TATAAA
		HSV-2 LAT TATA box mutant	at TAAt
ICP4-binding site	119532–119543	HSV-2 333	CGAACACAGGGG
		HSV-2 ICP4-binding site mutant	Ctcgag CAGGGG
miR-I	126732–126754	HSV-2 333	TTTGGAGTCTGCGGTTGGGAGC
		HSV-2 miR-I/II mutant	TcTgaGAaTcgGCaGTaGGCaC
miR-II-5p	126915–126935	HSV-2 miR-I/II rescuant	TTTGGAGTCTGCGGTTGGGAGC
		HSV-2 333	GAGTTCACTCGGCACGCATGC
miR-III	122988–123009	HSV-2 miR-I/II mutant	GAGTTCACT CaGatCtg ATGC
		HSV-2 miR-I/II rescuant	GAGTTCACTCGGCACGCATGC
		HSV-2 333	TCGCGCCTGCCCGGCCAGACT
		HSV-2 miR-III mutant	TCGCGCCTGCCCGGCC tAagga
		HSV-2 miR-III rescuant	TCGCGCCTGCCCGGCCAGACT

^aThe sequences of HSV-2 strain HG52 (GenBank accession no. [NC_001798](#)) were used as reference sequences.
^bSubstitution mutations are indicated with the corresponding lowercase, italic, and bold letters.

FIG 2 Northern hybridization to detect miRNAs from miRNA mutant viruses in productively infected Vero cells. (A) The same blot was probed first with the indicated probe for miR-I-5p and then stripped and probed with the indicated probe for miR-II-5p and subsequently with the probe for U6 snRNA. (B) An independent blot was probed first with a probe for miR-III and then stripped and probed with the probe for U6 snRNA expression. NIC, noninfected control; Res, rescuant. Illustrative diagrams for the pre-mRNAs, mature miRNAs, and probes used are shown to the right of the panels. Blue lines denote probes, red lines denote mature miRNA sequences, the X's denote mutations, and black lines denote pre-miRNA sequences. The locations of each pre-miRNA and of the expected miRNAs are indicated on each blot. 32 P-labeled 19-nucleotide (nt) and 24-nucleotide oligonucleotides were used as size makers. U6 independently probed on each blot was used as a loading control.

LAT TATA box mutant was significantly lower than that in those infected with the HSV-2 WT ($P = 0.01$) (Fig. 3C).

During the latent phase of infection from day 15 through day 42 p.i., the HSV-2 LAT TATA box mutant exhibited a WT recurrence phenotype ($P = 0.3$) (Fig. 3D). Thus, the HSV-2 LAT TATA box mutation did not alter elements that are essential for efficient recurrence in the guinea pig genital model of HSV-2 infection.

Lumbosacral DRGs from infected guinea pigs sacrificed during the latent phase of infection were evaluated for viral DNA and RNA levels. Viral DNA levels in the lumbosacral DRGs were not significantly different between the HSV-2 WT and the HSV-2 LAT TATA box mutant ($P = 0.4$) (Fig. 3E). The viral transcripts for LAT and ICP0 were also quantified in ganglia from infected guinea pigs by TaqMan quantitative reverse transcription-PCR (RT-PCR) assays and normalized to virus DNA levels (Fig. 3F). During latency, LAT and ICP0 RNA expression levels were similar between the viruses, with no statistically significant differences being observed ($P = 0.4$ for LAT and $P = 0.5$ for ICP0). Expression of the three most abundant HSV-2 LAT-region miRNAs, miR-I, -II, and -III, in ganglia from infected guinea pigs was quantified by TaqMan quantitative RT-PCR assays (Fig. 3G). During latency, the three RNA expression levels were similar between the viruses, with no statistically significant differences being observed ($P = 0.5$ for miR-I, $P = 0.7$ for miR-II, and $P = 0.9$ for miR-III), consistent with the finding of no difference in LAT expression.

The HSV-2 ICP4-binding site does not influence the recurrence phenotype in guinea pigs. To determine if the HSV-2 LAT ICP4-binding site is required for efficient reactivation of HSV-2 in guinea pigs, similar experiments and evaluations were performed using the HSV-2 WT and the HSV-2 LAT ICP4-binding site mutant. The identical mutation employed in the ICP4-binding site mutant virus was previously shown to prevent ICP4 repression of LAT transcription *in vitro* (3). The *in vitro* growth of the ICP4-binding site mutant virus was similar to that of wild-type HSV-2 (Fig. 4A). The mean lesion scores during the acute phase of infection were similar between the HSV-2

FIG 3 Evaluation of HSV-2 LAT TATA box mutant. (A) Northern hybridization of RNA from HEK 293 cells 48 h after transfection with pSSB, pSSB-Not⁻, and the pSSB-TATA mutant, using a LAT-specific double-stranded DNA probe. (B) *In vitro* growth curve in Vero cells infected at an MOI of 0.1, using WT and mutant viruses. (C) Mean lesion severity during acute infection in guinea pigs by time after infection with WT or mutant virus. (D) Mean number of cumulative recurrences per guinea pig for each group during latent infection. (E) Viral DNA quantities, as determined by TaqMan PCR and normalized to the level of the 18S rRNA gene, in the lumbosacral DRGs during latent infection. (F) Relative (to WT HSV-2-infected animals) LAT and ICP0 transcript expression in lumbosacral DRGs during latent infection (number of RNA copies/100 ng total RNA), normalized to the levels of 18S rRNA and virus DNA (as shown in panel E). (G) Relative miR-I, miR-II, and miR-III expression by RT-PCR normalized to the levels of 18S rRNA and virus DNA. Samples from animals with latent infection were harvested at day 71 postinoculation. The Mann-Whitney test was used for pairwise comparisons. *, $P < 0.05$.

WT and the mutant viruses ($P = 0.9$) (Fig. 4B). During the latent phase of infection, the HSV-2 LAT ICP4-binding site mutant had a WT recurrence phenotype ($P = 0.8$) (Fig. 4C). The region substituted in the HSV-2 LAT ICP4-binding site mutant thus also did not include elements that are essential for efficient recurrence in a genital model of HSV-2 infection.

Viral DNA levels in the lumbosacral DRGs during latency were not significantly different between the HSV-2 WT and the HSV-2 LAT ICP4-binding site mutant ($P = 0.1$) (Fig. 4D). During latency, virus DNA-normalized LAT and ICP0 RNA expression levels did not significantly differ between the viruses ($P = 0.6$ for LAT and $P = 0.9$ for ICP0), and the three miRNA expression levels were also similar between the viruses, with no statistically significant differences being observed ($P = 0.5$ for miR-I, $P = 0.6$ for miR-II, and $P = 0.4$ for miR-III) (Fig. 4E and F).

HSV-2 miR-I and miR-II do not influence the virus recurrence phenotype in guinea pigs. Similar experiments and evaluations were done using the HSV-2 WT and

FIG 4 Evaluation of HSV-2 LAT ICP4-binding site mutant. (A) *In vitro* growth curve in Vero cells infected at an MOI of 0.1, using WT and mutant viruses. (B) Mean lesion severity during acute infection in guinea pigs by time after infection with WT or mutant virus. (C) Mean number of cumulative recurrences per guinea pig for each group during latent infection. (D) Viral DNA quantities, as determined by TaqMan PCR and normalized to the level of the 18S rRNA gene, in the lumbosacral DRGs during latent infection. (E) Relative (to WT HSV-2-infected animals) LAT and ICP0 transcript expression in lumbosacral DRGs during latent infection (number of RNA copies/100 ng total RNA), normalized to the levels of 18S rRNA and virus DNA (as shown in panel D). (F) Relative miR-I, miR-II, and miR-III expression by RT-PCR, normalized to the levels of 18S rRNA and virus DNA. Samples from animals with latent infection were harvested at day 108 postinoculation. The Mann-Whitney test was used for pairwise comparisons. *, $P < 0.05$.

the HSV-2 miR-I/II mutant and its rescuant. Mutation of miR-I and of miR-II was previously shown to relieve miR-I and miR-II repression of ICP34.5 expression (26). *In vitro* growth curves were similar between the wild-type, mutant, and rescuant viruses (Fig. 5A). The mean lesion scores in guinea pigs infected with the HSV-2 miR-I/II mutant appeared to be lower than those in guinea pigs infected with the HSV-2 WT (though the difference was nonsignificant [$P = 0.06$]) and were not significantly lower than those in guinea pigs infected with the rescuant ($P = 0.2$) (Fig. 5B). During the latent phase of infection, animals infected with the HSV-2 miR-I/II mutant showed a reduced (though the difference was nonsignificant [$P = 0.06$]) recurrence rate compared with that for animals infected with the HSV-2 WT, but the recurrence phenotype in these animals was indistinguishable from that in animals infected with the rescuant ($P > 0.9$) (Fig. 5C). The region substituted in the HSV-2 miR-I/II mutant thus does not include elements required for recurrence in the context of the rescuant virus, which likely (together with the mutant) carried an additional attenuating mutation.

Viral DNA levels in the lumbosacral DRGs during latency of the HSV-2 miR-I/II mutant ($P = 0.03$) and the rescuant ($P = 0.03$) were significantly lower than those in the lumbosacral DRGs during latency of the HSV-2 WT (Fig. 5D), though those for the HSV-2 miR-I/II mutant and rescuant were not different from each other ($P = 0.2$). During latency, LAT and ICP0 RNA expression levels, adjusted for DNA quantity, were similar among the viruses, with no statistically significant differences being observed ($P \geq 0.2$ for all pairwise comparisons) (Fig. 5E), implying that the impaired acute-phase phenotype and reduced viral DNA levels in the lumbosacral DRGs of the mutant and rescuant cannot be attributed to the LAT or ICP0 expression capacity of these viruses. RT-PCR of RNA from latently infected lumbosacral DRGs yielded the expected finding that the miR-I expression level was significantly lower in the HSV-2 miR-I/II mutant than in the HSV-2 WT ($P = 0.03$) and the rescuant ($P = 0.03$). miR-II expression levels were also

FIG 5 Evaluation of the HSV-2 miR-I/II mutant. (A) *In vitro* growth curve in Vero cells infected at an MOI of 0.1, using WT and mutant viruses. (B) Mean lesion severity during acute infection in guinea pigs by time after infection with WT, mutant, or rescuant virus. (C) Mean number of cumulative recurrences per guinea pig for each group during latent infection. (D) Viral DNA quantities, as determined by TaqMan PCR and normalized to the level of the 18S rRNA gene, in the lumbar DRGs during latent infection. (E) Relative (to WT HSV-2-infected animals) LAT and ICP0 transcript expression in lumbar DRGs during latent infection (number of RNA copies/100 ng total RNA), normalized to the levels of 18S rRNA and virus DNA (as shown in panel D). (F) Relative miR-I, miR-II, and miR-III expression by RT-PCR, normalized to the levels of 18S rRNA and virus DNA. Samples from animals with latent infection were harvested at day 103 postinoculation. The Mann-Whitney test was used for pairwise comparisons. *, $P < 0.05$.

lower (though not significantly, likely due to assay background) in the HSV-2 miR-I/II mutant than in the HSV-2 WT ($P = 0.06$) or the rescuant ($P = 0.07$) but were similar between the WT and rescuant viruses ($P = 0.3429$). miR-III expression levels were similar among the viruses, with no statistically significant differences being observed ($P \geq 0.2$ for all pairwise comparisons) (Fig. 5F).

miR-III does not influence the HSV-2 recurrence phenotype in guinea pigs. To determine if HSV-2 miR-III is required for the efficient reactivation of HSV-2 in guinea pigs, similar experiments and evaluations were done using the HSV-2 WT or the HSV-2 miR-III mutant or its rescuant. The *in vitro* effect of miR-III on HSV-2 ICP0 expression was previously shown using synthetic miR-III mimics (26). The *in vitro* growth of wild-type, mutant, and rescuant viruses was comparable (Fig. 6A). The mean lesion scores were similar among the viruses ($P = 0.3$ for WT versus mutant, $P = 0.2$ for WT versus rescuant, and $P = 0.9$ for mutant versus rescuant) (Fig. 6B). During the latent phase of infection, the HSV-2 miR-III mutant and the rescuant had a WT recurrence phenotype ($P = 0.7$ for WT versus mutant, $P = 0.3$ for WT versus rescuant, and $P = 0.7$ for mutant versus rescuant) (Fig. 6C).

Viral DNA levels in the lumbar DRGs during latency were not significantly different among the HSV-2 WT, the HSV-2 miR-III mutant, and the rescuant ($P \geq 0.2$ for all pairwise comparisons) (Fig. 6D). During latency, LAT and ICP0 RNA expression was also similar among the viruses, with no statistically significant differences being observed ($P \geq 0.2$ for all pairwise comparisons) (Fig. 6E). Although there was some assay background, as expected for the miR-III deletion virus, miR-III expression was significantly lower in the HSV-2 miR-III mutant than in the HSV-2 WT ($P < 0.05$) and the

FIG 6 Evaluation of HSV-2 miR-III mutant. (A) *In vitro* growth curve in Vero cells infected at an MOI of 0.1, using WT and mutant viruses. (B) Mean lesion severity during acute infection in guinea pig by time after infection with WT, mutant virus, or rescuant virus. (C) Mean number of cumulative recurrences per guinea pig for each group during latent infection. (D) Viral DNA quantities, as determined by TaqMan PCR and normalized to the level of the 18S rRNA gene, in the lumbosacral DRGs during latent infection. (E) Relative (to WT HSV-2-infected animals) LAT and ICP0 transcript expression in lumbosacral DRGs during latent infection (number of RNA copies/100 ng total RNA), normalized to the levels of 18S rRNA and virus DNA (as shown in panel D). (F) Relative miR-I, miR-II, and miR-III expression by RT-PCR, normalized to the levels of 18S rRNA and virus DNA. Samples from animals with latent infection were harvested at day 78 postinoculation. The Mann-Whitney test was used for pairwise comparisons. *, $P < 0.05$.

rescued ($P = 0.008$), while miR-III expression was similar between the HSV-2 WT and the rescuant ($P > 0.9$). miR-I and miR-II expression levels during latency were similar among the viruses, with no statistically significant differences being observed ($P \geq 0.2$ for all pairwise comparisons) (Fig. 6F).

DISCUSSION

In the present study, mutant viruses were constructed and tested to evaluate specific hypotheses regarding the role of HSV-2 LAT promoter sequences and of the miRNAs expressed from the LAT in reactivation from viral latency. None of the mutant viruses had reactivation phenotypes that could be attributed to the mutations. We also did not observe any impact of these mutations on acute infections or establishment of viral latency, as determined by measurement of the total virus DNA detected in latently infected guinea pig lumbosacral ganglia.

While LAT promoter deletions of HSV-1 and HSV-2 have a diminished capacity for reactivation (11, 12), including in the guinea pig genital model, it is still not known why these deletion mutants have this phenotype. The discovery of LAT-associated miRNAs suggested a possible mechanism: by reducing ICP34.5 and ICP0 expression, the miRNAs might enhance establishment of latency during the acute infection and thus increase the amount of virus that is available to be reactivated at a later time (3, 9, 25–27). Mutation of miR-I and miR-II (which both target ICP34.5) together and of miR-III (which targets ICP0) separately did not influence the virus recurrence phenotype, indicating that the HSV-2 LAT promoter deletion phenotype in guinea pigs is not dependent on these factors (at least in isolation). In HSV-1, there appeared to be an increased ability of miRNA deletion mutants (in miR-H4 and miR-H3, homologs of miR-I and miR-II in HSV-2, respectively) to grow in cultured murine neuronal cells (25). We did not test this

specific finding but did not observe any differences among the mutants and controls with respect to acute infections either in cell culture or in animals. The LAT promoter deletion phenotypes in HSV-1 (in the rabbit eye model) (28) or in HSV-2 (in the guinea pig genital model) (12) appear not to be attributable to these miRNAs, which are capable of regulating ICP0 and ICP34.5 expression in mammalian cells.

In an attempt to identify possible combined effects of LAT-associated miRNAs, we introduced additional mutations in the LAT promoter that could have altered LAT expression and that thus could capture the effect of LAT-encoded miRNAs as a whole. However, in spite of demonstrated *in vitro* effects, specific mutations of the LAT TATA box and ICP4-binding site did not lead to an appreciable change in latent LAT or miRNA levels in animal experiments, suggesting that there may be additional promoter elements that also give rise to latent LAT and miRNA expression. Although the mutants with the LAT TATA and ICP4-binding site mutations showed the expected results in transfected human cells, it is also theoretically possible that these mutations were insufficient to completely eliminate the activity of these elements in guinea pigs *in vivo*. In HSV-1, sequences (called LAP2) downstream of the LAT promoter appear to have independent promoter activity (29). Sequences with similar activity have not been identified in HSV-2, although HSV-2 LAT exon 1 sequences do appear to have enhancer activity (20).

The LAT TATA box mutant virus appeared to cause weaker acute infections *in vivo* than its parent. Because the recurrence phenotype was similar to that in the parent and our goal was to study the effect of these mutations on reactivation, we did not construct a rescuant to determine whether these milder acute infections were due to the LAT TATA box mutation or an inadvertently introduced mutation elsewhere in the virus.

Mutation of miR-I, miR-II, and miR-III sequences abolished expression of miR-III and of the pre-miRNA for miR-I and changed the sizes of the pre-miRNA for miR-II and miR-III in cell cultures infected with the corresponding miRNA mutant viruses. While PCR detection of miRNAs is often associated with some background (where a weakly positive signal is present even when the miRNA is not), we were able to show a >1-log reduction of both mutated miRNAs in the miR-I/miR-II mutant *in vivo*. The miR-III PCR assay showed less background, also permitting a clear demonstration that the mutation abolished miR-III expression *in vivo*. The reduced frequency of recurrence observed with the miR-I/miR-II mutant was not restored in a rescuant of this virus, implying the presence of another mutation that reduced the capacity of both this mutant virus and the rescuant to reactivate. Thus, the appropriate comparison is between the recurrence phenotype of the mutant and the rescuant, indicating that in the context of other viral sequences present in the rescuant, the miR-I/miR-II mutations did not influence the recurrence phenotype. The miR-III mutation also did not influence the recurrence phenotype.

Mutation of the LAT promoter/enhancer or LAT-associated miRNAs evaluated in this study did not identify a specific role for these sequences in the reactivation phenotype in guinea pigs. Other sequences in this region, potentially including those associated with another transcript transcribed from the opposite strand (antisense to LAT) in the LAT promoter region (the primary transcript for miR-H6, which also, in isolation, appears not to be responsible for the LAT promoter deletion phenotype) (30), may also play a role in the LAT-associated phenotype in animals. However, these findings do not rule out a role of miRNA or LAT promoter sequences in human latency or reactivation.

MATERIALS AND METHODS

Cells, viruses, and plasmids. Vero cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and maintained in minimum essential medium with 10% inactivated fetal bovine serum (FBS) (HyClone) and 1% penicillin-streptomycin-L-glutamine (Quality Biologicals, Gaithersburg, MD). HSV-2 strain 333 as the WT originally was obtained from Gary Hayward (Johns Hopkins University, Baltimore, MD). Plasmid clones of an SphI-BamHI fragment (Sph-Sal-Bam plasmid [pSSB]) and an SphI-KpnI fragment (SphI-Sall-KpnI plasmid [pSSK]) of HSV-2 strain 333 were described previously (20, 26). pSSB-Not⁻, which contains a 624-bp NotI fragment deletion spanning the LAT promoter and part of LAT exon 1, was also described previously (12). The pSSB-TATA mutant, containing a 3-bp mutation of

the LAT TATA box in which TATAAA was mutated to atTAAt (AseI digestion site), was constructed by subcloning a synthesized mutant DNA fragment containing the NotI-NotI sequences (Integrated DNA Technologies, Coralville, IA) into pSSB using the NotI site; substitution mutations are indicated with the corresponding lowercase letters. The pSSB-LAT ICP4-binding site mutant plasmid containing a 5-bp mutation at the HSV-2 LAT transcription initiation (and ICP4-binding) site, in which CGAACA was mutated to Ctcgag (XhoI digestion site), was constructed by PCR using pSSB as the template with the primers oFP1 (CCCCTTTCGCGCCTTTCGCGAA) and oST518 (CAGCAACtcgagCAGGGGGCCGCGCCGAT) or oBP3 (GCGAGCCGGGACAGATGCGGA) and oST517 (CCCCTGcctcgaGTTGCTGCCGCGCCCCGG) to yield overlapping fragments containing the mutant LAT ICP4-binding site. A mixture of these purified PCR fragments was used as the template and reamplified with oFP1 and oBP3. The resulting PCR product was digested with NotI and subsequently cloned into pSSB between the NotI sites.

PCR fragments containing mutations in miR-I, miR-II, and miR-III were constructed using overlapping PCR. A miR-I mutant PCR fragment was constructed using primers oST395 (GGAAAAGAGCGGGGGCGG GAGTCC) and oST426 (GGTTCAACCCCTAGACCGCCCGACGG) and purified PCR products from primer sets oST395 with oST402 (cTGaGAaTCgGCaGTaGGcAaCGCGCCGGGGCGCGGCACG) and oST426 with oST399 (GtTgCcTAcTGCcGATcTcAgATGGTCCCTGCGTACGACTCGGG) with pSSK as the template. The miR-II mutant PCR fragment was constructed using primers oST500 (TCCTGCCGCGCCCTTAAGAG) and oST426 and purified PCR products from primer sets oST500 with oST522 (GTTCACTCaGatCtgATGCACG TGTAACCGCCAGT) and oST426 with oST521 (TGCATCaGatCtGAGTGAACCTCTCCGCCCCGA) with the miR-I mutant PCR fragment as the template. The miR-III mutant PCR fragment was constructed using primers oST537 (GTCGTGCCGAGAGTGGCCTCTCTT) with oST538 (CGGCCTGGGACGACGGAGA) and purified PCR products from primer sets oST537 with oST540 (GCcTAggaCTGTGCTTGGGTGTCGGTCTGA) and oST538 with oST539 (ACAGtcctTaGGCCGGGCGAGGCGCGA) with pSSB as the template. The mutant viruses were produced by homologous recombination in cotransfected Vero cells, as previously described (12), between an excess of plasmid or PCR fragment bearing the mutation with flanking virus sequences as described above and purified virus genomic DNA. Where relevant, rescuant viruses of miR-I/II or miR-III were constructed by cotransfection of purified mutant virus with pSSB or pSSK. Plaques were screened by PCR or Southern hybridization and were purified two additional times after screening indicated the absence of the parent virus. All mutations were verified by sequencing of the mutated region of each mutant virus. Viral stocks were produced in Vero cells. To compare the growth characteristics of WT and mutant viruses, 10^6 Vero cells were inoculated in duplicate at time zero with a multiplicity of infection of approximately 0.1 PFU/cell of each virus. Medium was added after a 2-h adsorption period. At 0, 2, 5, and 16 h postinoculation (p.i.), cells were scraped, freeze-thawed three times, and titrated in duplicate by plaque assay.

Transfection and Northern hybridization. HEK 293 cells (6×10^5) were transfected in six-well plates with 3 μ g of the plasmids indicated in Fig. 2A using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were harvested at 48 h after transfection. After total RNA was extracted, 5 μ g was subjected to electrophoresis through a denaturing formaldehyde–1.5% agarose gel as previously described (20). After transfer and immobilization to a Nytran membrane, RNAs were hybridized with a radiolabeled gel-purified Sall-XhoI fragment that overlaps the LAT intron and ICP0 3' end labeled with [α - 32 P]dCTP using a Prime-aGene labeling system (Promega, Madison, WI) in Hybrisol I hybridization solution (EMD Millipore Corporation, Temecula, CA) at 56°C overnight. The membrane was then washed twice with 6 $\times$ SSPE (1 $\times$ SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA [pH 7.7]), 0.5% SDS for 15 min at room temperature, twice with 1 $\times$ SSPE, 0.5% SDS for 15 min at 37°C, and once with 0.1 $\times$ SSPE, 0.1% SDS for 30 min at 65°C. Vero cells were infected with the miR-I-II mutant, the miR-I-II-mutant rescuant, the miR-III mutant, the miR-III-mutant rescuant, or wild-type HSV-2 strain 333 at a multiplicity of infection (MOI) of 2. Total RNAs were collected at 20 h postinfection and separated on a 12% urea-PAGE gel before transfer to a GeneScreen Plus nylon membrane (PerkinElmer). The probes and Northern hybridization conditions used for the detection of miR-I, miR-II, and miR-III have been previously described (9, 26).

Animal studies. Female Hartley guinea pigs (Charles River, Wilmington, MA) were inoculated intravaginally with 2×10^5 PFU of each virus. Guinea pigs were monitored and scored daily during acute infection (14 days) for lesion severity (on a scale of from 0 to 4; 0 = no disease, 1 = redness/swelling, 2 = 1 to 2 lesions, 3 = 3 to 5 lesions, and 4 = 6 or more lesions or the coalescence of lesions). Recurrences, defined as new vesicular lesions, were enumerated during the latent phase from day 15 through day 42 after inoculation and graphed as cumulative recurrences per guinea pig in each group. All observations of guinea pigs were made by two independent investigators masked to the identity of the inoculated viruses, and composite scores are presented in the figures. Animals were housed in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care and cared for in accordance with Food and Drug Administration guidelines. Lumbosacral (L1 through S2) DRG tissues were collected from each animal immediately after sacrifice (which occurred from days 71 to 108 but which was performed on the same days for animals in each individual experiment and was performed only on animals with no visible external recurrences for at least 2 days prior to sacrifice), snap-frozen on dry ice, and stored at -80°C . DNA and RNA were isolated from ganglion tissues using an AllPrep DNA/RNA minikit (Qiagen, Valencia, CA) after homogenization with a tissue homogenizer, Precellys 24 (Bertin Instruments, Montigny-le Bretonneux, France). In addition, sequences of the appropriate viruses were recovered from the ganglia of latently infected animals infected with each virus. The relevant region of each virus was amplified by PCR from virus stocks and from guinea pig lumbosacral DRGs in each animal experiment using relevant primers (oFP1 and oBP3 for the LAT TATA box and the LAT ICP4-binding site, oST395 and oST426 for miR-I, oST500 and oST426 for miR-II, and oST537 and

oST538 for miR-III). PCR products were cloned into the pCR4-TOPO vector, and selected colonies were sequenced using M13 primers (Life Technologies, Carlsbad, CA). The sequences verified the presence of the expected wild-type, mutant, or rescuant virus and did not indicate the presence of unexpected viruses.

Detection of viral DNA and transcripts by real-time PCR and single-step RT-PCR. The copy numbers of viral DNA genomes and RNA transcripts in DRGs were determined by quantitative real-time PCR (for DNA) and quantitative reverse transcription-PCR (RT-PCR) (for RNA) using a QuantStudio 6 Flex real-time PCR system (Applied Biosystems, Foster City, CA), using previously described primers (31), with slight modifications to the template, primer, and probe concentrations or cycling conditions being made to accommodate changes in real-time PCR instrumentation. The forward primer at 50 nM, reverse primer at 300 nM, and probe at 100 nM were used for DNA quantification using the LAT-based PCR. The forward primer at 400 nM, reverse primer at 400 nM, and probe at 100 nM were used for LAT RNA quantification. The forward primer at 400 nM, reverse primer at 900 nM, and probe at 100 nM were used for ICP0 RNA quantification. Quantitative PCR was performed with TaqMan Universal PCR MasterMix (Applied Biosystems, Foster City, CA) and 250 ng of DNA as the template, and quantitative RT-PCR was performed with TaqMan Fast Virus one-step master mix (Applied Biosystems, Foster City, CA) and 100 ng of total RNA as the template. Cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min for the real-time PCR and 50°C for 5 min and 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s, for real-time RT-PCR. For the detection of viral DNA and RNA, all results were normalized to those for 18S rRNA (DNA and RNA, respectively), and transcript quantities were further adjusted by viral DNA copy numbers. Because infected cells were used as the RT-PCR standard, RT-PCR did not provide absolute results; therefore, RT-PCR results are presented relative to the quantity of each viral RNA found in guinea pigs infected with wild-type HSV-2 in the same experiment.

Detection of virally encoded miRNAs by real-time PCR. Methods for the detection of virally encoded miRNAs by real-time PCR were previously described (9, 26), but the primer and probe concentrations were slightly modified to accommodate a change in instrumentation. One hundred nanograms of total RNA was used as the template for all reactions, and the volume of all reaction mixtures was 20 μ l. For miR-I detection, reverse transcription (primer concentration, 0.8 nM) was performed prior to PCR with 50 nM an miR-I-specific TaqMan probe, 300 nM forward primer, and 120 nM reverse primer. For miR-II detection, the reverse transcription primer concentration was 1.5 nM. Cycle conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 63°C for 1 min.

Statistics. The Mann-Whitney test and other statistical tests were performed using GraphPad Prism (version 6.02) software for Windows (GraphPad Software, La Jolla, CA). Comparisons of acute infections were based on total areas under the lesion severity curves for days 1 to 14 after the inoculations. Comparisons of recurrent infections were based on the number of cumulative recurrences per guinea pig adjusted for the number of days of observation. Error bars represent standard errors of the means for each group.

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