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MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs

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Abstract

Herpesviruses are characterized by their ability to maintain life-long latent infections in their animal hosts. However, the mechanisms that allow establishment and maintenance of the latent state remain poorly understood. Herpes simplex virus 1 (HSV-1) establishes latency in neurons of sensory ganglia, where the only abundant viral gene product is a non-coding RNA, the latency associated transcript (LAT)[1,2]. Here, we show that LAT functions as a primary microRNA (miRNA) precursor that encodes four distinct miRNAs in HSV-1 infected cells. One of these miRNAs, miR-H2-3p, is transcribed antisense to ICP0, a viral immediate-early transcriptional activator thought to play a key role in productive HSV-1 replication and reactivation from latency[3]. miR-H2-3p is indeed able to reduce ICP0 protein expression, but does not significantly affect ICP0 mRNA levels. We also identified a fifth HSV-1 miRNA in latently infected trigeminal ganglia, miR-H6, which derives from a previously unknown transcript distinct from LAT. miR-H6 displays extended seed complementarity to the mRNA encoding a second HSV-1 transcription factor, ICP4, and inhibits expression of ICP4, which is required for expression of most HSV-1 genes during productive infection[4]. These results may explain the reported ability of LAT to promote latency[5-9]. Thus, HSV-1 expresses at least two primary miRNA precursors in latently infected neurons that may facilitate the establishment and maintenance of viral latency by post-transcriptionally regulating viral gene expression.

HSV-1 LAT is an ~8.3 kb capped, polyadenylated RNA (Fig. 1a)[1,2] that is spliced to give an ~2.0 kb stable intron and a predicted unstable ~6.3 kb exonic RNA[10,11]. As LAT is not thought to encode a protein, we hypothesized that the exonic regions of LAT might function as a primary miRNA precursor[12]. To identify HSV-1 LAT-derived miRNAs, we constructed a LAT expression plasmid, pcDNA3/LAT, in which a heterologous promoter drives transcription of an ~10.8 kb HSV-1 genomic fragment containing the entire 8.3 kb LAT (Fig. 1a). We transfected this plasmid into human 293T cells and isolated total RNA. Northern analysis revealed high-level expression of the stable LAT intron (Fig. 1b).

Small RNAs derived from this sample were used to prepare cDNAs for 454 sequencing[13]. This resulted in 225,439 sequence reads (Suppl. Table 1), of which at least 144,955 represented cellular miRNAs (Suppl. Table 2A). We also recovered 651 HSV-1-derived miRNAs (Suppl. Tables 1 and 3). Six HSV-1 miRNA sequences were obtained, derived from four HSV-1 miRNA precursor hairpins (Fig. 2a). The two most common HSV-1 miRNAs were miR-H2-3p

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(265 reads) and miR-H4-3p (266 reads) and these derived from miRNA stem-loops that also gave rise to star strands miR-H2-5p (10 reads) and miR-H4-5p (61 reads) (Fig. 2a). We also detected miR-H3 (5 reads) and miR-H5 (40 reads). For each miRNA, HSV-1 LAT could be folded into the expected precursor stem-loop structure. Where both the miRNA and star strand were recovered, the characteristic ~2 nt 3′ overhangs were observed in the duplex intermediate (Fig. 2a).

These data show that LAT can be processed into miRNAs in culture but do not address expression *in vivo*. We therefore isolated small RNAs from trigeminal ganglia (TG) of mice latently infected with HSV-1 and performed deep sequencing of derived cDNAs. We obtained 254,651 sequence reads (Suppl. Table 1), of which at least 204,867 represent cellular miRNAs (Suppl. Table 2B). An additional 164 sequences represented HSV-1 miRNAs (Suppl. Tables 1 and 4). miR-H2-3p (94 reads), miR-H3 (18 reads) and miR-H5 (1 read) represent LAT-derived miRNAs previously identified in LAT-expressing 293T cells (Fig. 2a). However, a fourth HSV-1 miRNA, miR-H6 (50 reads), derives from an RNA stem-loop transcribed from the opposite strand of the HSV-1 genome, within the LAT promoter (Fig. 1a). This sequence was not present in pcDNA3/LAT and therefore could not be detected in transfected 293T cells. Of the total of 171 HSV-1 short RNAs detected in TG, 27 were obtained only once. Of these, 20 represent truncations or point mutants of miR-H2 through miR-H6, while 7 appear to represent random HSV-1 RNA breakdown products (Suppl. Table 1 and data not shown).

The identification of miR-H6 is striking for two reasons. Firstly, miR-H6 must derive from a second HSV-1 primary miRNA precursor, distinct from LAT, expressed in latently infected neurons. While a transcript antisense to the LAT promoter has been described[14], the reported ends of this transcript exclude miR-H6. The lack of previous reports describing this primary miRNA precursor may reflect the fact that it must be cleaved to generate miR-H6, and hence is likely unstable. Secondly, the stem-loop that gives rise to miR-H6 lies antisense to a stem-loop transcribed from the opposite DNA strand that gives rise to a previously described HSV-1 miRNA, miR-H1 (Fig. 2a). miR-H1 is expressed late in productive replication,[15] and miR-H6 and miR-H1 show extensive sequence complementarity (Fig. 2b). The unusual phenomenon of distinct miRNAs derived by bidirectional transcription of a single genomic locus was recently also described in mouse cytomegalovirus[16].

To ascertain whether any of these HSV-1 miRNAs are expressed during productive HSV-1 infection, where LAT is expressed late in infection[11], we performed quantitative stem-loop RT-PCR for miR-H2-3p through miR-H6 using RNA from HSV-1 infected Vero cells. The cellular miRNA let-7a was used as an internal control for RNA recovery. All five novel HSV-1 miRNAs were, in fact, detected in infected Vero cells using RT-PCR (Fig. 2c and Suppl. Table 5A) and/or Northern analysis (Suppl. Fig. 2d). The “non-LAT” HSV-1 miRNA miR-H6 was detected at $10^{5.0}$ molecules per ng of isolated short (i.e., <200 nt) RNA, while the four LAT-derived miRNAs were detected at between $10^{2.7}$ (miR-H3) and $10^{4.1}$ (miR-H2-3p) molecules per ng (Fig. 2c and Suppl. Table 5A). These data confirm that all five novel HSV-1 miRNAs are indeed expressed in productively infected cells.

RT-PCR analysis of pcDNA3/LAT-transfected 293T cells (Fig. 2c) also detected all four LAT-derived miRNAs, but as expected did not detect miR-H6, which is not present in this vector. Analysis of short RNAs derived from mouse TG demonstrated the expression of all four LAT-derived HSV-1 miRNAs, as well as miR-H6 (Fig. 2c and Suppl. Table 5A). There is a relatively poor correlation between the levels of expression of each HSV-1 miRNA, as extrapolated from deep sequencing, when compared to the qRT-PCR analysis. This presumably reflects differences in the efficiency of cDNA synthesis.

The qRT-PCR analysis presented in Fig. 2c and Suppl. Table 5A allows us to roughly estimate how many copies of each HSV-1 miRNA are present in productively infected Vero cells versus latently infected neurons. During productive infection, miR-H1 and miR-H6 are expressed at ~1200 and ~300 copies per Vero cell. In contrast, the LAT-derived HSV-1 miRNAs miR-H2-3p through miR-H5 are all present at <40 copies per cell (Suppl. Table 6A). These latter levels may be too low to exert a significant phenotypic effect. In latently infected TG, our estimate is based on a previous report that mice latently infected with the HSV-1 strain KOS contain ~500 LAT-expressing neurons per TG[17]. Based on this report, we estimate ~6.3×10^4 copies per LAT+ neuron for miR-H2-3p, ~4×10^4 copies/LAT+ neuron for miR-H6 and ~8×10^5 copies/LAT+ neuron for miR-H4-3p. We also detected substantial levels of miR-H4-5p (~3.2×10^4 copies/LAT+ neuron), thus suggesting that the star strand of miR-H4 might also be a functional miRNA (Fig. 2c and Suppl. Table 6C). Even if our estimate of the number of latently HSV-1 infected neurons per TG is low by an order of magnitude[18], the level of HSV-1 miRNAs per neuron would still be within the range of cellular miRNAs that is biologically active[12].

Although we were able to detect several different HSV-1 miRNAs in both LAT-expressing 293T cells and infected Vero cells, using a range of techniques, we did not detect the previously described miR-LAT[19] (Suppl. Figs. 1 and 2). The report describing miR-LAT was recently retracted.

Mapping of the six HSV-1 miRNAs onto the HSV-1 genome reveals that miR-H2 is antisense to the ICP0 transcript, while both miR-H3 and miR-H4 are antisense to ICP34.5 (Fig. 1a). ICP0 is an HSV-1 transcriptional activator, expressed as an immediate-early gene, that promotes viral replication and may facilitate reactivation from latency[3,20,21]. To examine whether miR-H2-3p could affect ICP0 protein or mRNA expression, we transfected 293T cells with either a wildtype ICP0 expression plasmid or a derivative containing three point mutations within the predicted miR-H2-3p seed region (Fig. 3a). These plasmids were co-transfected with plasmids designed to express an shRNA that mimics the predicted miR-H2 pre-miRNA (Fig. 2a and Suppl. Fig. 3) or a mutated version of the miR-H2 pre-miRNA (miR-H2/3M) that bears three mutations in the miR-H2-3p seed region that restore complementarity to the ICP0 mutant (Fig.3a). As shown in Fig. 3b, the wildtype miR-H2 pre-miRNA inhibited expression of wildtype, but not mutant, ICP0 protein. Conversely, expression of the mutant ICP0 protein was reduced upon co-expression of the mutant miR-H2/3M pre-miRNA but was not affected by wildtype miR-H2. While these data demonstrate that miR-H2-3p is indeed acting through the expected target site to inhibit ICP0 protein expression, this inhibition did not correlate with a reduction in the level of ICP0 mRNA (Fig. 3c). Similar data, obtained using siRNA duplexes designed to mimic the miR-H2 or miR-H2/3M miRNA duplex intermediate, and using RNAse protection to measure ICP0 mRNA expression, are presented in Suppl. Fig. 3. Together, these data show that, despite the perfect complementarity of miR-H2-3p to ICP0 mRNA, inhibition of ICP0 protein expression by this viral miRNA occurs primarily at the translational level[12]. These data are consistent with earlier reports suggesting that LAT reduces ICP0 protein, but not mRNA, levels in infected cells.[22,23]

Analysis of other HSV-1 genes revealed sequence similarity between miR-H6, including an extended miRNA seed region,[12] and the mRNA encoding ICP4, a transcription factor required for expression of most HSV-1 genes during productive infection (Fig. 4a)[4]. Co-transfection of an ICP4 expression plasmid with a synthetic form of the predicted miR-H6 duplex intermediate revealed strong downregulation of ICP4 protein expression (Fig. 4b), while an ICP4 expression construct with three mutations in the seed region of the predicted miR-H6 target site remained unaffected. Analysis of wildtype ICP4 mRNA expression levels showed that miR-H6 co-expression had little or no inhibitory effect (Fig. 4C).

In this manuscript, we report the identification of five novel HSV-1 miRNAs, three of which were previously computationally predicted[15,24]. Four of these viral miRNAs derive from the second exon of the spliced ~6.3 kb LAT (Fig. 1a) and these miRNAs may provide both a rationale for the existence of spliced LAT and explain its characteristic instability[1,11], i.e., LAT is likely degraded in the nucleus due to Drosha cleavage[12]. In addition to the four LAT-derived HSV-1 miRNAs, we also identified a fifth miRNA, miR-H6, derived from a currently unknown primary miRNA precursor that lies antisense to the LAT promoter and that must also be expressed in latently infected neurons (Fig. 1a). Of interest, miR-H6 lies antisense to a known late HSV-1 miRNA, miR-H1[15].

Three of the latently expressed HSV-1 miRNAs are transcribed antisense to HSV-1 mRNAs —*ICP0* mRNA in the case of miR-H2-3p and *ICP34.5* mRNA in the case of both miR-H3 and miR-H4-3p (Fig. 1a)—and we have demonstrated that miR-H2-3p is indeed able to inhibit ICP0 protein expression (Fig. 3b). As ICP0 is a key immediate-early HSV-1 transcriptional activator that may promote entry into the productive replication cycle[3,20,21], inhibition of ICP0 expression by miR-H2-3p may increase the likelihood that neurons enter and maintain latency. It has in fact been previously proposed that LAT inhibits ICP0 expression post-transcriptionally in neurons[2,10,23] and the existence of miR-H2-3p could explain this phenomenon. We also observed that miR-H6 displays partial complementarity to ICP4 mRNA, including an extended miRNA seed region[12], and can reduce ICP4 protein expression (Fig. 4). Like ICP0, ICP4 can promote exit from latency[21], and inhibition of ICP4 expression may therefore enhance the robustness of the latent state.

While we have not directly examined the effect of miR-H3 and miR-H4-3p on ICP34.5 expression, it appears likely that these viral miRNAs are also acting as inhibitors of viral gene expression. Data favouring this hypothesis come from analysis of the L/ST transcripts that overlap the 3′ end of LAT (Fig. 1a). L/ST RNAs are expressed by HSV-1 mutants lacking ICP4[25]. Importantly, the L/ST RNAs, which have the potential to give rise to miR-H3 and miR-H4-3p (Fig. 1a), are known to inhibit ICP34.5 expression via an “antisense” mechanism[26,27] and these viral miRNAs are presumably responsible for this effect. In conclusion, our observation that HSV-1 miRNAs are capable of downregulating key viral immediate early proteins is consistent with the recent proposal, based primarily on computational data, that herpesviruses in general may use viral miRNAs “as part of their strategy to enter and maintain latency.”[28]

Methods Summary

pcDNA3/LAT expresses an ~10.8 kb EcoRV to BamHI fragment, derived from the KOS strain of HSV-1, which extends 134 bp 5′, and ~2.3 kb 3′, to LAT. Small RNAs were prepared using standard techniques from 293T cells transfected with pcDNA3/LAT or from the dissected TG of mice latently infected with HSV-1 strain KOS 30 days previously. cDNAs were prepared and subjected to 454 sequencing[13]. Vero or SY5Y cells were infected with HSV-1 strain KOS or strain 17syn+ at 10 pfu/cell and RNA harvested for RT-PCR analysis 14 to 18 hrs post-infection. Northern and Western blot analyses were performed using standard methods. Stem-loop RT-PCR methods are described in supplementary materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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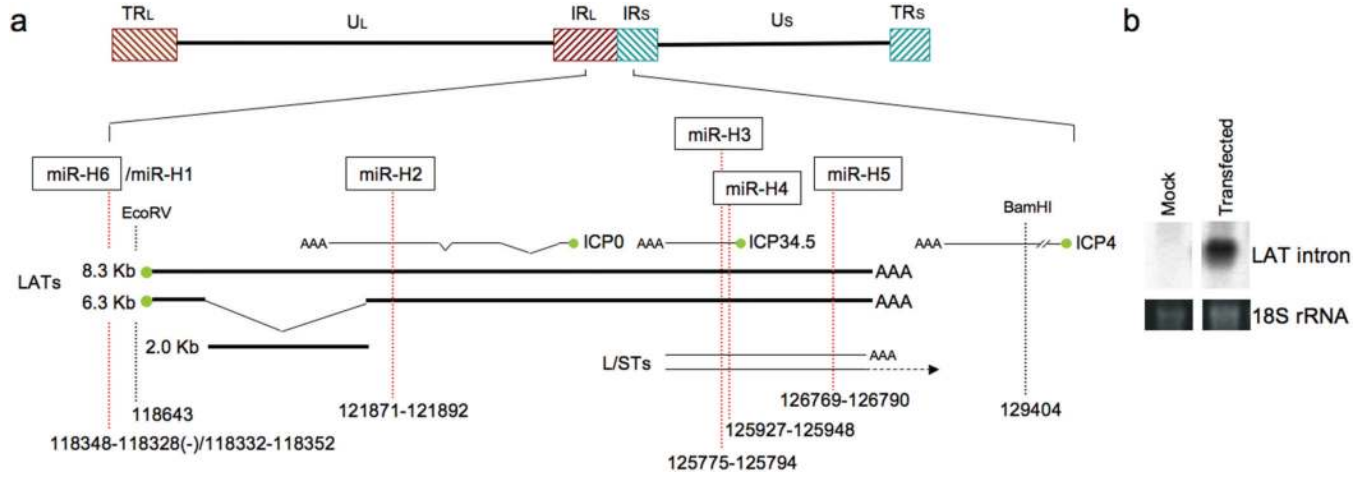

Fig.1.

Genomic location of HSV-1 miRNAs. a. Schematic of the HSV-1 genome expanded to display details of the LAT locus. Relative sizes, locations and orientations of other viral transcripts in this region are indicated. Sequence coordinates of viral miRNAs and restriction enzyme sites are given according to the HSV-1 strain 17 syn+ genome (NC_001806). All viral miRNAs are in the same orientation as LAT except for miR-H6. An EcoRV-BamHI fragment containing LAT was cloned into pcDNA3, to generate pcDNA3/LAT. TR, terminal repeat; IR, internal repeat; U_L, unique long; U_S, unique short. b. Northern blot for the ~2.0 kb LAT intron, demonstrating LAT expression after transfection of pcDNA3/LAT into 293T cells. The lower bands show 18S rRNA, a loading control. Small RNAs from this sample were used for cDNA preparation and 454 sequencing.

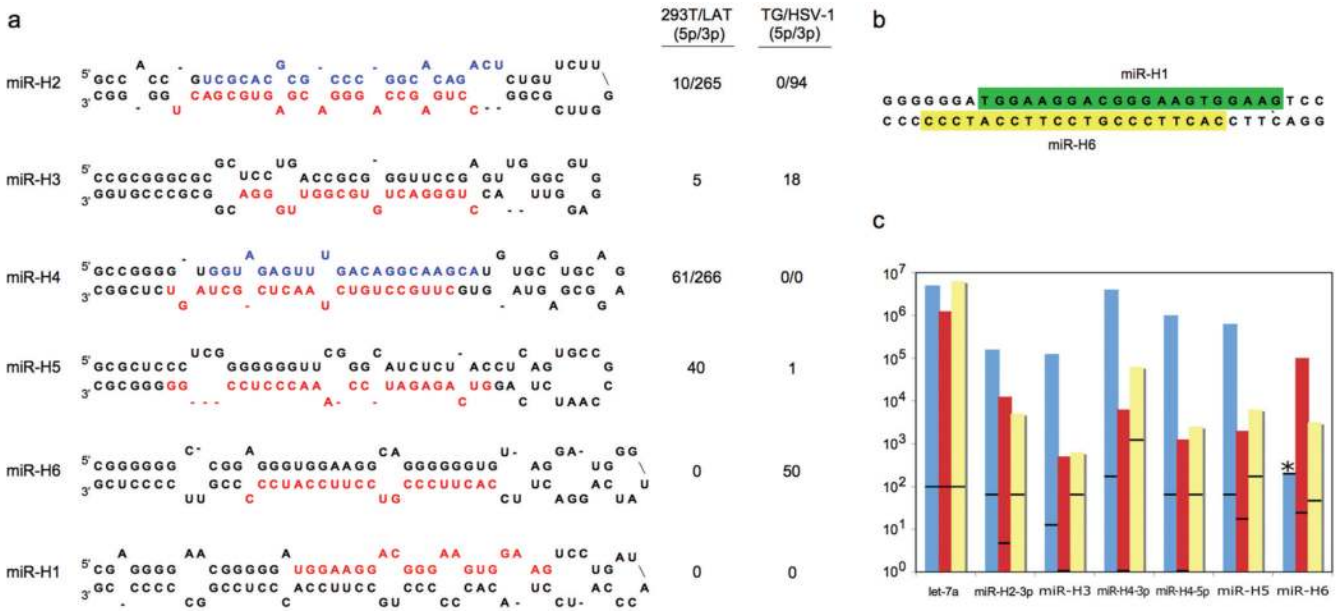

Fig. 2.

2HSV-1 pre-miRNAs. a. Predicted secondary structures of HSV-1 miRNA precursors, demonstrating the characteristic stem-loops. Mature miRNAs are indicated in red and, where observed, star strands in blue. Number of reads of each recovered mature miRNA sequence are indicated. Where the star strand was also obtained, these are given as 5p/3p. miR-H1 and miR-H6 were not recovered from transfected 293T cells because pcDNA3/LAT lacks these sequences. b. HSV-1 genomic sequence showing the antisense orientation and overlap of mature miR-H6 and the predicted sequence of miR-H1[15]. c. Quantitative RT-PCR analysis verifying the existence and relative expression of HSV-1 miRNAs in 293T cells transfected with pcDNA3/LAT (Tx, blue), Vero cells infected with HSV-1 (red), or mouse TG latently infected with HSV-1 (yellow). miRNA abundances are displayed as copies per ng of short-enriched RNAs (<200 nts). Horizontal lines indicate background levels for each miRNA assayed. *; not detected. See Suppl. Table 5 for relevant controls.

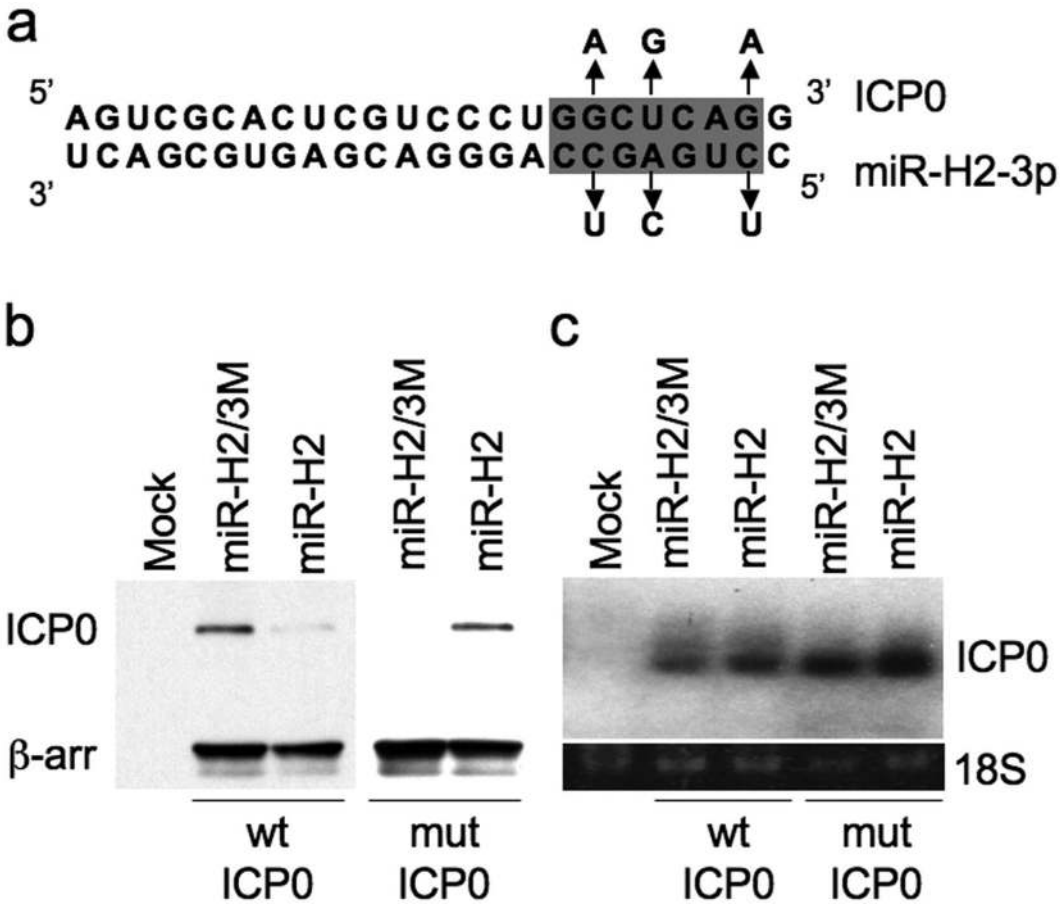

Fig. 3.

Downregulation of ICP0 protein expression by HSV-1 miR-H2-3p. a. Sequence of miR-H2-3p bound to ICP0 mRNA. The miRNA seed region is indicated in gray. Arrows indicate complementary nucleotide changes introduced into the mutant ICP0 expression plasmid and miR-H2-3p/3M expression construct. b. Western blot analysis of ICP0 protein expression. 293T cells were transfected with a β-arrestin expression plasmid and either a wildtype or mutant ICP0 expression construct. Plasmids expressing either miR-H2-3p or miR-H2-3p/3M were co-transfected. c. Northern analysis of the samples shown in panel b. 18S rRNA was used as a loading control.

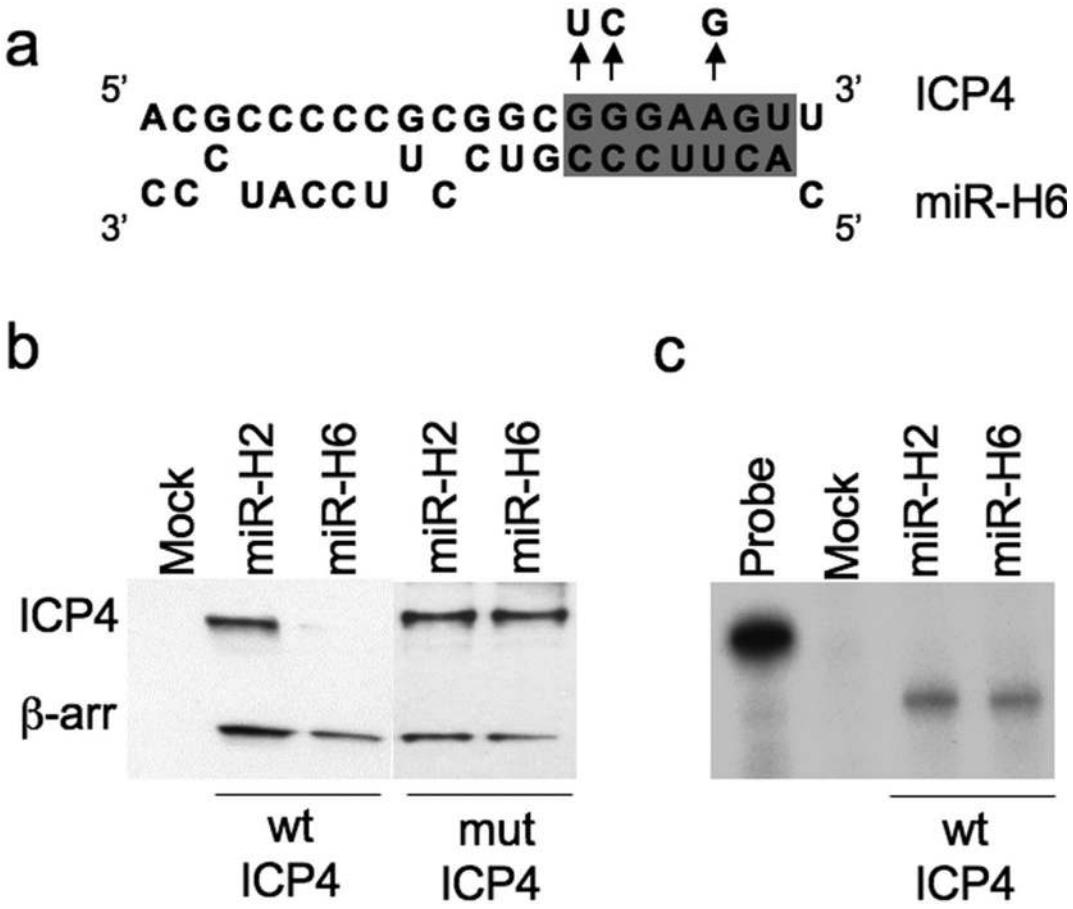

Fig. 4.

Downregulation of ICP4 protein expression by HSV-1 miR-H6. a. Sequence complementarity of miR-H6 to nucleotides 127,298 to 127,319 of the ICP4 mRNA. Grey box indicates the miRNA seed region. Arrows indicate nucleotide changes present in the ICP4 mutant. b. Western blot analysis of ICP4 protein expression. 293T cells were co-transfected with a synthetic miR-H6 duplex intermediate and plasmids expressing either wildtype ICP4 or the ICP4 mutant. A miR-H2 duplex intermediate served as a negative control, as ICP4 mRNA has no predicted target sites for miR-H2-3p. c. RNAse protection analysis of ICP4 mRNA levels in the wildtype ICP4 samples shown in panel b.