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# A microRNA of infectious laryngotracheitis virus can downregulate and direct cleavage of ICP4 mRNA

Lisa A. Waidner [a], Joan Burnside [b], Amy S. Anderson [b], Erin L. Bernberg [b], Marcelo A. German [c], Blake C. Meyers [d], Pamela J. Green [d], Robin W. Morgan [b,*]

[a] Elcriton, Inc. 15 Innovation Way, Suite 288, Newark, DE 19711, USA
[b] Department of Animal and Food Sciences, Delaware Biotechnology Institute, University of Delaware, Newark, DE 19711, USA
[c] Dow AgroSciences, LLC, 16160 SW Upper Boones Ferry Road, Portland, OR 97224, USA
[d] Department of Plant and Soil Sciences, Delaware Biotechnology Institute, University of Delaware, Newark, DE 19711, USA

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ABSTRACT

Viral microRNAs regulate gene expression using either translational repression or mRNA cleavage and decay. Two microRNAs from infectious laryngotracheitis virus (ILTV), iltv-miR-I5 and iltv-miR-I6, map antisense to the ICP4 gene. Post-transcriptional repression by these microRNAs was tested against a portion of the ICP4 coding sequence cloned downstream of firefly luciferase. Luciferase activity was downregulated by approximately 60% with the iltv-miR-I5 mimic. Addition of an iltv-miR-I5 antagomiR or mutagenesis of the target seed sequence alleviated this effect. The iltv-miR-I5 mimic, when co-transfected with a plasmid expressing ICP4, reduced ICP4 transcript levels by approximately 50%, and inhibition was relieved by an iltv-miR-I5 antagomiR. In infected cells, iltv-miR-I5 mediated cleavage at the canonical site, as indicated by modified RACE analysis. Thus, in this system, iltv-miR-I5 decreased ILTV ICP4 mRNA levels via transcript cleavage and degradation. Downregulation of ICP4 could impact the balance between the lytic and latent states of the virus *in vivo*.



## Introduction

MicroRNAs are small, non-coding RNAs found in plant, animal, and viral genomes. These molecules are considered important contributors to regulation of cellular processes because of their widespread and diverse tissue- and condition-specific expression profiles (He and Hannon, 2004) and because they can regulate at least 60% of vertebrate transcripts (Friedman et al., 2009). MicroRNAs are generally thought to function in animal cells by base-pairing to their target mRNAs, which leads to translational inhibition or mRNA degradation (Bartel, 2004; Filipowicz et al., 2008; Nilsen, 2007). Complementarity between nucleotides 2 and 7/8 of the microRNA (known as the "seed" sequence) is crucial for target recognition in mRNA destabilization, translational repression, or both, and 100% complementarity is important for RNA-induced silencing complex (RISC)-mediated cleavage (Bartel, 2009). Some microRNAs act as small interfering RNAs (siRNAs) through an RNAi mechanism via 100% complementarity to their targets (Wang et al., 2004). A number of viruses, herpesviruses in particular, encode microRNAs (Cullen, 2006; Pfeffer et al., 2005). Because of the compact nature of viral genomes, many viral microRNAs are derived from non-coding transcripts antisense to functional genes. However, regardless of genomic address, viral microRNAs can act via an siRNA mechanism (Barth et al., 2008; Seo et al., 2008, 2009; Sullivan et al., 2005; Tang et al., 2008, 2009) or via imperfect base pairing to targets, as with animal microRNAs (Grey et al., 2007; Murphy et al., 2008; Umbach et al., 2008).

Herpesviruses, which are characterized by latent infections, are likely to use microRNAs to enhance viral effects on host cells, and this can be accomplished by regulating either host or viral genes (Cullen, 2009). Epstein-Barr virus (EBV) (Pfeffer et al., 2004), Kaposi's sarcoma-associated herpesvirus (KSHV) (Cai et al., 2005), human herpes simplex virus 1 (HSV1) (Umbach et al., 2008), murine herpesvirus 68 (MHV68) (Pfeffer et al., 2005), human cytomegalovirus (HCMV) (Grey et al., 2005), Marek's disease virus 1 (MDV1) (Burnside et al., 2006; Yao et al., 2008), MDV2 (Waidner et al., 2009; Yao et al., 2007), herpesvirus of turkeys (HVT) (Waidner et al., 2009), and infectious laryngotracheitis virus (ILTV) (Waidner et al., 2009) are among the herpesviruses known to encode microRNAs. Some viral microRNAs are expressed during latency and therefore may be important in regulating the balance between productive and latent viral states. For instance, microRNAs of MDV1 (Burnside et al., 2006), MDV2 (Waidner et al., 2009; Yao et al., 2007), EBV (Grundhoff et al., 2006; Pfeffer et al., 2004), KSHV (Cai et al., 2005; Grundhoff et al.,

* Corresponding author. University of Delaware, Delaware Biotechnology Institute, Room 245, 15 Innovation Way, Newark, DE 19711, USA. Fax: +1 302 831 4054.
*E-mail address:* morgan@udel.edu (R.W. Morgan).

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2006; Pfeffer et al., 2005; Samols et al., 2005), HSV1 (Umbach et al., 2008), Rhesus monkey rhadinovirus (RRV) (Schafer et al., 2007), and murine herpesvirus 68 (MHV68) (Pfeffer et al., 2005) are predominantly clustered at one or two sites in the genome, and one of the clusters lies in a region active during latency. In some cases, microRNAs are antisense to sequences within (Waidner et al., 2009; Yao et al., 2007) or in the vicinity of (Burnside et al., 2006; Yao et al., 2008) the immediate early gene, ICP4.

Many viral microRNAs map antisense to viral lytic genes, but the mechanism of downregulation of the antisense targets varies among and within viruses. Viral microRNAs that are highly complementary to their targets may regulate them at the mRNA level, at the protein level, or not at all. MicroRNA-mediated cleavage is used in regulation of the large T-antigen mRNA in two human polyomaviruses (JC and BK) (Seo et al., 2008) and murine polyoma virus (Sullivan et al., 2009). Additionally, HSV2-miR-H3 is 100% complementary to the neurovirulence factor gene ICP34.5 and downregulates it in an siRNA-like manner (Tang et al., 2008). In contrast, translation inhibition is used by HSV1-miR-H2, which maps antisense to the immediate-early transactivator ICP0 gene (Umbach et al., 2008). In HCMV, miR-112-1 has high complementarity (15/22 nucleotide match) to the immediate early gene IE1 but does not affect mRNA levels even though it does decrease IE1 protein expression (Grey et al., 2007; Murphy et al., 2008). HSV miRs H3 and H4 are 100% complementary to ICP34.5, but regulation of this transcript by these microRNAs has not been addressed (Umbach et al., 2008). However, HSV miR-H6 shows extensive seed complementarity to the HSV ICP4 and does downregulate expression of ICP4 (Umbach et al., 2008).

ILTV is an alphaherpesvirus belonging to the genus *Iltoviridae*. It has a general genome structure that resembles that of HSV, but unlike most alpha-herpesviruses, it does not contain the repeat regions flanking the unique long genome segment ($I/TR_L$). ILTV causes a respiratory disease in chickens and pheasants, with the severity of the disease varying from decreased egg production to conjunctivitis and severe tracheal infections resulting in bloody expectoration (Bagust et al., 2000; Garcia and Riblet, 2001; Kirkpatrick et al., 2006). In addition, after a short acute infection phase of 2 weeks or less, the virus becomes latent in the central nervous system (Williams et al., 1992) and can subsequently reactivate, resulting in re-infection of exposed flocks (Hughes et al., 1989; Hughes et al., 1991). The most abundant microRNAs from ILTV, iltv-miR-I5 and iltv-miR-I6, map antisense to the ICP4 gene (Rachamadugu et al., 2009; Waidner et al., 2009), which is located in the internal and terminal repeats flanking the unique short genome segment ($I/TR_S$). ICP4 is a major transcriptional regulator that is essential for viral growth; it is an immediate-early gene that is repressed during latency. In the study presented here, we investigate whether iltv-miR-I5 could downregulate the immediate early gene ICP4.

## Results

### *iltv-miR-I5 reduced expression from reporter plasmids containing complementary sites*

The action of iltv-miR-I5 and iltv-miR-I6 on the expression of ILTV ICP4 was tested using luciferase reporter assays. A 409-bp fragment from the coding region of ICP4, corresponding to nucleotides 115,017–115,425 in the $IR_S$ (146,290–146,708 in the $TR_S$), was cloned downstream of the luciferase gene in the pMIR-REPORT™ vector (pMiR-Report-I5-I6-target, Fig. 1, inset). This portion of the IPC4 open reading frame contains sequences that are 100% complementary to iltv-miR-I5 and iltv-miR-I6. Plasmid pMiR-Report-I5-I6-target was co-transfected into COS7 cells along with mimics and antagomiRs. Transfections included the pMIR-REPORT™ β-galactosidase plasmid to evaluate transfection efficiency, and relative luciferase activity was determined (Fig. 2). The addition of the iltv-miR-I5 mimic resulted in significant repression ($p<0.05$), with approximately 60% reduction in luciferase activity compared to control samples (Fig. 2A). Repression of luciferase activity was observed for the iltv-miR-I6 mimic but was not significant (23% reduction, $p=0.072$). Samples co-transfected with a combination of mimics for iltv-miR-I5 and iltv-miR-I6 showed a level of luciferase repression similar to that seen with iltv-miR-I5 alone (Fig. 2A), and this was also significantly lower than the negative control ($p<0.05$).

To confirm the specificity of the negative regulatory effect of iltv-miR-I5, we mutated three nucleotides in the seed target region for iltv-miR-I5 in plasmid pMiR-Report-I5-I6-target (Fig. 2B). The iltv-miR-I5 mimic significantly reduced expression of the wild-type target construct by approximately 60% and was significantly different from the control ($p<0.05$, Fig. 2C), and the addition of an antagomiR of miR-I5 alleviated this effect ($p=0.49$). Co-transfection of the iltv-miR-I5 mimic with the mutated construct did not result in a significant reduction (15% reduction, $p=0.21$, Fig. 2C). As expected, the co-transfection with the mutant construct along with the iltv-miR-I5 and its antagomiR did not differ significantly from the control transfection ($p=0.11$).

In order to assess whether physiological levels of ILTV-derived miR-I5 could downregulate ICP4, iltv-miR-I5 target luciferase constructs were transfected into LMH cells infected with ILTV. The timing of ILTV growth and microRNA expression was first tested in this cell culture system with an ILTV multiplicity of infection (MOI) of 2.2 (Supplemental Fig. 1). A qPCR based on quantification of the gC gene was used to measure total viral genomes per well. In this system, the number of ILTV genomes per well was maximal at 35–50 h post-infection (hpi) (Supplemental Fig. 1A). In addition, iltv-miR-I5 was measured by Northern analysis, and microRNA accumulation was detected as early as 19 hpi (Supplemental Fig. 1B) but was higher at later times (28 and 50 hpi). A 331-bp fragment containing the iltv-miR-I5 target region in the coding region of ICP4 (nucleotides 146,391–146,708 in the $TR_S$) was cloned downstream of the luciferase gene in the pMIR-REPORT™ vector (pMiR-Report-I5-target, Fig. 1, inset). Luciferase constructs WT pMiR-Report I5-target, its mutated derivative, or the empty vector pMIR-REPORT™ were transfected at 36.5 to 49.5 hpi. At 4.5 to 9 h post-transfection, cells were assayed for normalized luciferase values. In numerous experiments, both wild-type and mutated target constructs were expressed at lower levels compared to expression in cells transfected with empty vector pMIR-REPORT™ (data not shown). This finding was unexpected but reproducible, and it could indicate that a cellular microRNA or even another regulatory mechanism can target ILTV ICP4 independent of iltv-miR-I5.

The experimental protocol was modified such that the cell culture medium was not removed during the transfection process in order to allow maximum accumulation of virus and virus-encoded microRNAs. Data from the two experiments done with this modified protocol are shown in Fig. 3. Both wild-type and mutated targets were expressed at lower levels compared to expression in cells transfected with empty vector. In mock-infected cells, there was no significant difference between the wild-type and mutant reporter constructs. In the ILTV-infected cells, there was a slightly lower expression (16–17% difference) of the wild-type compared to the mutant reporter (Fig. 3), and the value obtained from the wild-type transfection was significantly lower than that of the mutated construct in both experiments ($p<0.05$). However, the effect was not large, the experimental protocol was delicate to optimize, and the assay was complicated by the background reduction of reporter expression in mock-infected cells.

### *iltv-miR-I5 reduced ICP4 mRNA levels by transcript cleavage*

Since iltv-miR-I5 is 100% complementary to the coding region of ICP4, we hypothesized that this microRNA could act as an siRNA and

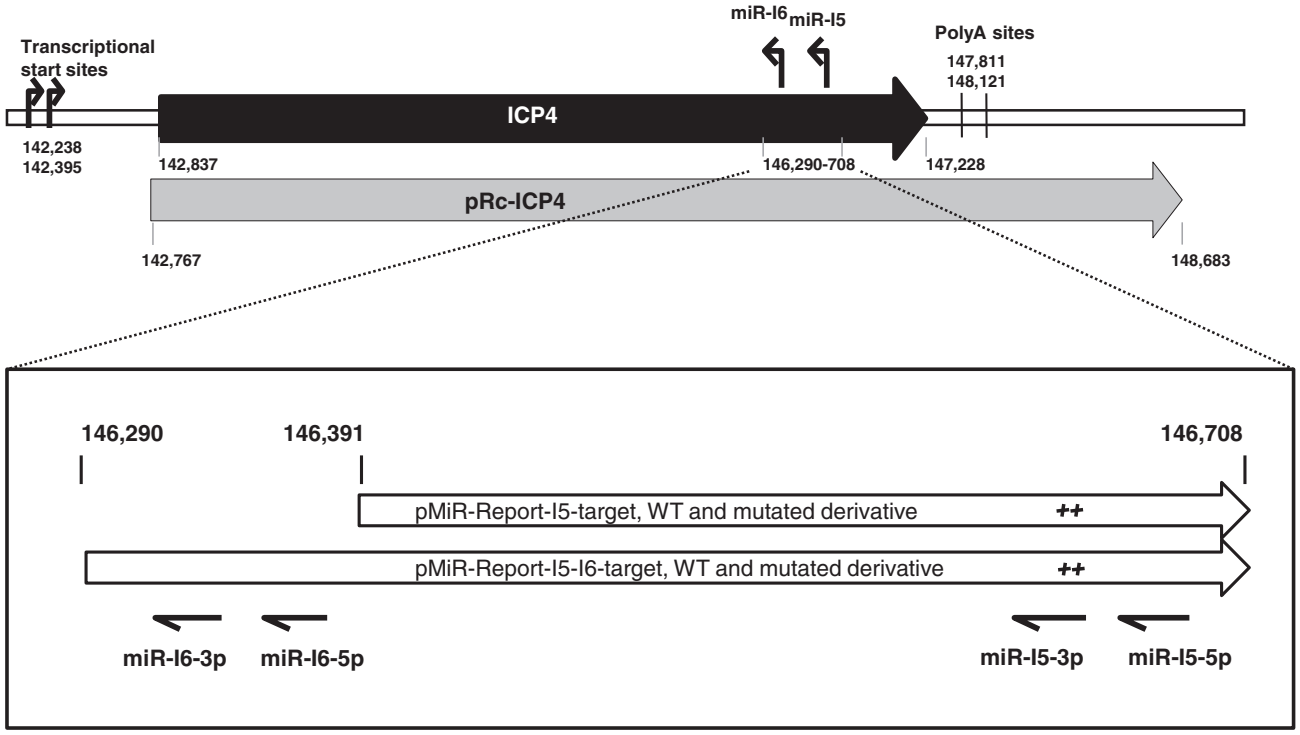


**Fig. 1.** Map of regions of the ILTV genome used in this study. The open reading frame of the ICP4 gene is indicated by the large black arrow. The ILTV microRNAs iltv-miR-I5 and iltv-miR-I6 antisense to the ICP4 coding region are represented by small black arrows. The portion of the genome containing the open reading frame of ICP4 cloned in pRc-ICP4 (Fuchs et al., 2000) is shown with the grey arrow. Predicted transcriptional start and polyA signals for ICP4 (Johnson et al., 1995) are indicated. Inset: a diagram of the inserts of luciferase constructs is shown in the box. Wild-type pMiR-Report-I5-I6-target (409 bp) and its mutated derivative were used in COS7 luciferase assays in Fig. 2. Wild-type pMiR-Report-I5-target (331 bp) and its mutated derivative were used in luciferase assays in ILTV-infected LMH cells, shown in Supplemental Fig. 2. The locations of the single nucleotide mutations in the seed complement in the mutated derivatives of the pMiR-Report-target constructs are denoted by black plus signs.

direct cleavage of the ICP4 transcript. We examined ICP4 mRNA levels in LMH cells co-transfected with a plasmid construct expressing ICP4 (pRc-ICP4) (Fuchs et al., 2000) along with microRNA mimics and antagomiRs (Fig. 4). Total RNA was subjected to quantitative RT-PCR (qRT-PCR) using a primer pair designed to amplify the region of the ICP4 coding region that contains the putative binding site for iltv-miR-I5 (5T-F and 5T-R; Supplemental Fig. 2A). All ICP4 RT-PCR reactions were normalized to cellular GAPDH. Transfection of iltv-miR-I5 resulted in approximately 50% reduction in RNA levels compared to the control, and this reduction was significant ($p<0.01$). Transfection with iltv-miR-I5 plus its antagomiR alleviated this effect. The iltv-miR-I6 mimic did not result in apparent reduction of ICP4 mRNA in this assay (Supplemental Fig. 2B). When primers flanking an area downstream of the potential target regions for iltv-miR-I5 and miR-I6 were used, no significant downregulation was observed in treatments containing microRNA mimics miR-I5 or miR-I6 (Supplemental Fig. 2). These data are consistent with the idea that the area downstream of the putative microRNA cleavage site is protected from degradation after cleavage (Llave et al., 2002).

To verify that the cleavage products were generated specifically by iltv-miR-I5, a modified RACE procedure that captures endonuclease-generated mRNA fragments with 5′ phosphorylated ends, which are suitable for ligation with T4 RNA ligase (Franco-Zorrilla et al., 2007; German et al., 2008; Llave et al., 2002), was used for locating the cleavage sites. Primary and secondary RACE amplifications were expected to result in 267 and 212-bp amplicons, respectively, since the reverse RACE primers (Supplementary Table 1) map to those genomic locations downstream of the predicted cleavage site for iltv-miR-I5 (genome position 146,630). To test the RACE procedure, we first prepared RNA from COS7 cells co-transfected with pRc-ICP4 and either the iltv-miR-I5 mimic or with the negative control mimic (negative control mimics from Ambion, Inc., # AM17110). RACE amplicons of the expected size, indicating the adapter was ligated to the predicted cleavage site at nucleotide #11 of the microRNA, were observed in the sample transfected with the iltv-miR-I5 mimic, but no product was seen in the negative control mimic sample (data not shown). To establish that iltv-miR-I5 acts as an siRNA in an ILTV infection, the RACE procedure was repeated on RNA samples isolated from ITLV-infected LMH cells. The ICP4 cleavage products were cloned and sequenced (Fig. 5). Eighteen clones were sequenced, and six clones mapped to the region of ICP4 surrounding the presumed binding site for iltv-miR-I5 (Fig. 5). For four of those six, sequence analysis indicated that the cleavage site was between nucleotides #10 and 11 of the iltv-miR-I5 binding site (Fig. 5). Two clones mapped nearby, but outside of the iltv-miR-I5 binding site, 13 and 28 nucleotides upstream of iltv-miR-I5. The remaining 12 clones all mapped to the open reading frame of UL45 at genome position 24,259 or 24,245, near the ILTV origin of replication (data not shown).

## Discussion

This study demonstrates that an ILTV microRNA that is antisense to the major transcriptional activator ICP4 has the potential to act as an siRNA and direct cleavage of the ICP4 transcript. Using luciferase reporters and qRT-PCR, iltv-miR-I5 repressed ICP4 by about 50%, whereas iltv-miR-I6 was less effective. The mutation of three nucleotides within the iltv-miR-I5 seed recognition sequence removed the effect of iltv-miR-I5 on luciferase activity. In addition, a modified RACE procedure validated that cleavage could occur within the predicted binding site.

Herpesvirus microRNAs appear to be related to latency associated transcript (LAT) expression and/or processing. Indeed, iltv-miR-I5 and iltv-miR-I6 could even be derived from processing of the putative ILTV LAT (Johnson et al., 1995). In the case of MDV1, the LAT transcript is antisense to ICP4 and generally shows inverse levels of expression with this gene (Cantello et al., 1994; Cantello et al., 1997). Transcription from the MDV1 LAT region is complex, and microRNAs

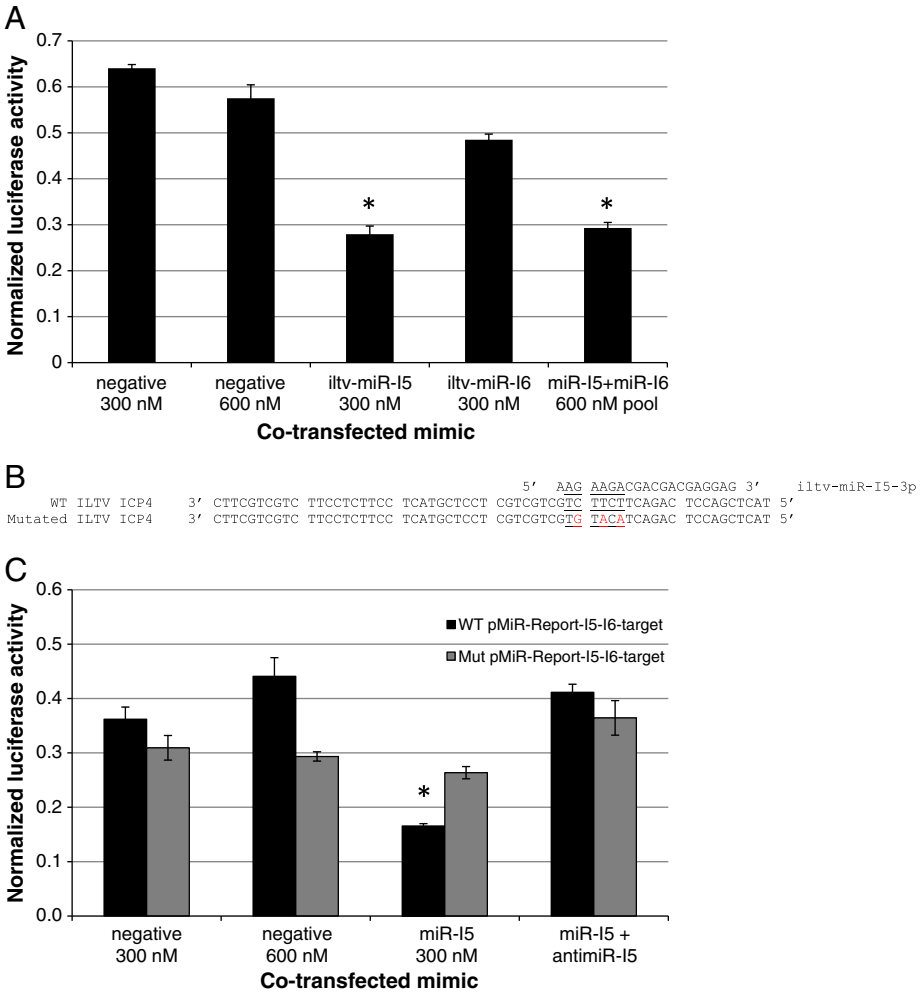


**Fig. 2.** The iltv-miR-I5 inhibits expression of a reporter mRNA containing the target regions for iltv-miR-I5 and iltv-miR-I6. (A) COS7 cells were co-transfected with 300 nM microRNA mimics (iltv-miR-I5, -miR-I6, or negative control) or 600 nM mimic pool (iltv-miR-I5 plus its antagomiR) and the luciferase reporter plasmid, pMiR-Report-I5-I6-target, plus a transfection control plasmid (pMIR-REPORT™ β-galactosidase.) For each treatment, three independent transfections were performed. (B) The predicted iltv-miR-I5 binding site within the ICP4 coding region is shown aligned with the iltv-miR-I5. The mutated nucleotides in pMiR-Report-MUT-I5-I6-target are shown in red. (C) The effects of the iltv-miR-I5 mimic and an antagomiR specific for iltv-miR-I5 (antimiR-I5) were examined following co-transfection with target plasmids pMiR-Report-I5-I6-target or its mutated derivative. Asterisks denote a significant difference ($p<0.05$) from the 300 nM or 600 nM negative control treatments.

may be derived from the LAT and its processed products (Burnside et al., 2006; Morgan et al., 2001), suggesting that LAT-derived microRNAs could play a role in maintaining latency or controlling the balance between productive and latent viral states. Although some studies indicate that the LAT has no effect on latency in HSV 1 (Block et al., 1990; Ho and Mocarski, 1988; Sedarati et al., 1989), other studies indicate that it is important in the establishment of latency (Perng et al., 2000; Sawtell, 1997), and improper splicing of the LAT affects the reactivation kinetics of HSV1 in neuronal tissues (Kang et al., 2003). The HSV1 LAT encodes numerous microRNAs, some of which are expressed during latency, suggesting that LAT-derived microRNAs are important in the lytic/latent balance of HSV1 (Jurak et al., 2010; Umbach et al., 2008). Understanding the relationship between herpesvirus microRNAs and LATs is likely to be a key to discovering regulatory details that dictate host–virus interactions.

In general, when microRNAs mediate mRNA target cleavage, perfect complementarity between the microRNA and its target exists (Bartel, 2004; Friedman et al., 2009). This mechanism is typically used in plants but can also occur in animals. The Bartel laboratory reported the cloning of a mouse HOXB8 mRNA fragment containing near-perfect complementarity to miR-196, with cleavage between the tenth and eleventh nucleotides of the microRNA (Yekta et al., 2004). A number of herpesviruses encode microRNAs that show 100% complementarity with viral genes and regulate gene expression by an siRNA-like mechanism (Barth et al., 2008; Seo et al., 2008, 2009; Sullivan et al., 2005; Tang et al., 2008; Tang et al., 2009). There are exceptions to this, as many microRNAs lie antisense to and are exactly complementary to their targets but do not direct cleavage of the putative mRNA targets. For example, the HCMV uracil DNA glycosylase protein activity is downregulated by miR-UL112-1 (Stern-Ginossar et al., 2009), but the mRNA apparently is not cleaved by this microRNA (Grey et al., 2008). Other studies of virally encoded microRNAs suggest that those antisense to viral genes do not always participate in mRNA degradation in the experimental systems used (Murphy et al., 2008; Umbach et al., 2008), and multiple mechanisms may exist for viral microRNA action.

In our study, iltv-miR-I5 was more effective than iltv-miR-I6 at downregulating ICP4, although both are antisense to ICP4. In chicken embryo kidney cells (CEK) infected with ILTV (4 h post-infection), iltv-miR-I5 was the most abundant viral microRNA (Waidner et al., 2009). However, in LMH or CEK cells 3 days post-infection, the dominant ILTV microRNA was iltv-miR-I6 (Rachamadugu et al., 2009). Thus, the levels of expression of different microRNAs could vary with the viral replicative cycle, although a side-by-side evaluation of the expression kinetics of these microRNAs has not been made. Even if iltv-miR-I5 and iltv-miR-I6 were derived from the same transcript,

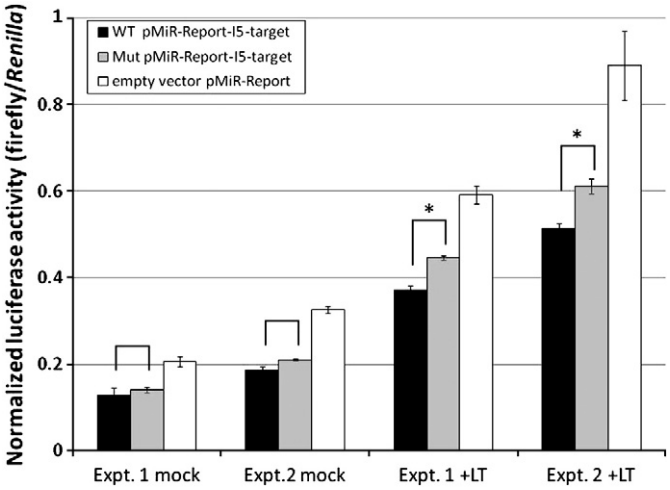


**Fig. 3.** Wild-type and mutated luciferase constructs transfected into ILTV-infected LMH cells. In two experiments, LMH cells were infected with ILTV at an MOI of 0.22 (Experiment 1) or 0.16 (Experiment 2), and infection was allowed to progress 37 h (Expt 1) or 38 h (Expt 2). Cells were then transfected with luciferase constructs containing no insert (empty vector pMiR-Report), or constructs containing inserts with wild-type (WT) or mutated (Mut) portions of the LTV ICP4 gene. Plasmid pRL-CMV (*Renilla* luciferase) was included in all samples as the transfection control. In both experiments, at 7 h post-transfection, cells were assayed for firefly and *Renilla* luciferase activities, and normalized luciferase values were calculated. Bars (±SE) are the average of three biological replicates. Asterisks denote a significant difference ($p<0.05$) between the wild-type (WT) and mutant (Mut) target constructs.

variation in processing and stability could produce different levels of these microRNAs, as has been noted for other polycistronic microRNAs (Sempere et al., 2004; Yao et al., 2008). A thorough analysis of microRNA expression through lytic and latent cycles or an examination of relative microRNA abundances in latently infected tissues such as trigeminal ganglia could provide additional insight into the different roles of these two microRNAs.

An alternative explanation for the differential activity by iltv-miR-I5 and iltv-miR-I6 is the secondary structure of the target, as has been suggested previously for *C. elegans* and human microRNAs (Long et al., 2007; Obernosterer et al., 2008). We performed *in silico* folding of the 800-nucleotide region containing the targets for iltv-miR-I5 and iltv-miR-I6. The secondary structure patterns in all possible foldings indicate that the iltv-miR-I6 binding region lies within a well-protected stable stem-loop, while the iltv-miR-I5 binding region is folded into a stem-loop containing a predicted three-way structure (Supplemental Fig. 3).

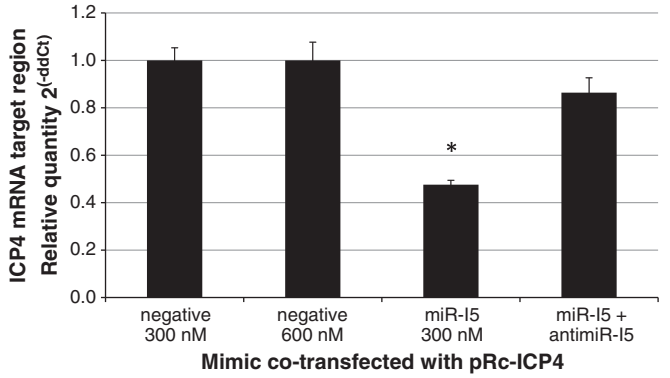


**Fig. 4.** Quantitative RT-PCR analysis of the effect of ILTV microRNA mimics on ICP4 mRNA levels. LMH cells were co-transfected with a plasmid expressing ICP4 (pRc-ICP4) and 300 nM microRNA mimics (iltv-miR-I5 or negative control) or 600 nM mimic pool (iltv-miR-I5 plus its antagomiR). Total RNA was subjected to qRT-PCR for the ICP4 gene, normalized to cellular GAPDH, and the values are expressed as relative quantities ($2^{-ddCt}$). Error bars represent standard error for three PCR replicates. Asterisks denote a significant difference ($p<0.05$) from the 300 nM or 600 nM negative control treatments.

In the case of iltv-iltv-miR-I6 and others (Grey et al., 2007; Murphy et al., 2008; Umbach et al., 2008), stable secondary structures within potential target sites may preclude microRNA binding and subsequent target downregulation (Obernosterer et al., 2008), whether it be via translational repression or mRNA cleavage. Additionally, the introduction of cell-type-specific or sequence-specific cofactors could affect microRNA function by blocking accessibility of the mRNA target molecule (Bartel, 2009; Hobert, 2008).

MicroRNA regulation of ICP4 could impact the balance between lytic and latent states, aid in maintaining latency of the virus *in vivo*, or play a role in virus reactivation from latency. Our study adds to other recent work that highlights the role of viral microRNAs in the regulation of viral immediate early proteins. These data suggest that iltv-miR-I5 could function in the regulation of latency by downregulation of targets through the siRNA slicing mechanism. The differential effects of antisense microRNAs iltv-miR-I5 and iltv-miR-I6 in this study emphasize the likely importance of other factors, such as target secondary structure, in microRNA-mediated mRNA cleavage.

## Materials and methods

### *Cell cultures and viruses*

The ILTV USDA strain was obtained from ATCC and is maintained in the University of Delaware collection. The COS7 (Gluzman, 1981) and LMH cell lines (Kawaguchi et al., 1987) were obtained from the University of Georgia and University of Delaware collections. All cells were grown in DMEM with 10% fetal bovine serum (FBS, Mediatech, Inc.) and 1% penicillin–streptomycin–neomycin (PSN) antibiotic mixture (Invitrogen, Inc.). LMH cells were plated on tissue culture vessels coated with 1% porcine gelatin (Sigma).

### *Luciferase assays*

COS7 cells were co-transfected with pMIR-REPORT™ vector (Ambion, Inc.) constructs (pMiR-Report-I5-I6-target and its mutated derivative, Fig. 1) and control pMIR-REPORT™ β-galactosidase (Ambion) along with microRNA mimics (Ambion, Inc.) and antagomiRs (Exiqon, Inc.). Negative control mimics from Ambion, Inc. (AM17110) contained no known homology to the chicken genome. Transfections were done in 96-well plates with Lipofectamine 2000 using the rapid transfection method according to the manufacturer's instructions. Briefly, 10,000 to 25,000 cells were mixed with nucleic acid and lipid complexes, containing 8 ng/μl of each reporter plasmid and either 300 nM or 600 nM mimics, and plated directly in DMEM with 10% FBS and no antibiotics. Cells were allowed to attach and replicate for 24–48 h. The Dual-Light® Combined Reporter Gene Assay System (Invitrogen, Inc.) was used to determine relative levels of β-galactosidase and firefly luciferase according to the manufacturer's instructions. Normalized firefly luciferase values were determined by addition of substrates and measurement of luminescence for 0.1 s in a Wallac Victor3 plate reader (Perkin Elmer). Student's *t*-test (two-tailed) was performed to compare treatment normalized luciferase values to those of either 300 nM or 600 nM negative control mimic treatments.

### *Luciferase assays in infected LMH cells*

LMH cells were seeded in 96-well plates in DMEM supplemented with 2% fetal bovine serum (FBS) and no antibiotics. Cells were subsequently infected with ILTV USDA strain, at MOIs ranging from 0.16 or 0.22, and infection was allowed to progress 37–38 h. The media was not removed prior to transfection, to ensure that virally derived microRNAs and virus remained in the culture medium throughout the course of the experiment. Cells were then transfected with luciferase constructs containing no insert (pMiR-Report empty

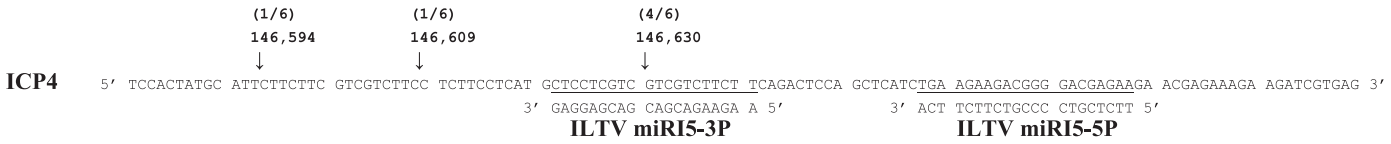


**Fig. 5.** Modified 5′ RACE for the detection of iltv-miR-I5 cleavage products. A portion of the ICP4 coding region, which maps antisense to iltv-miR-I5-5p and iltv-miR-I5-3p, is shown aligned with the microRNAs. The three positions of RACE cleavage fragments are indicated with grey highlighting and arrows, and the numbers of clones in parentheses and genome positions are shown above the sequence.

vector), or constructs containing inserts of the portion of the ILTV ICP4 open reading frame complementary to the iltv-miR-I5 microRNA. Inserts contained either the wild-type (WT) or mutated (Mut) seed sequence complements (pmiR-Report-I5-Target, WT and Mut, Fig. 1). Each treatment was also co-transfected with plasmid pRL-CMV (Renilla luciferase, Promega) as the transfection control. Transfections were done with Lipofectamine 2000 using the standard method according to the manufacturer's instructions. Briefly, nucleic acid and lipid complexes were formed, containing 50 ng target plasmid, 50 ng transfection control plasmid, and 0.25 μl Lipofectamine 2000 and incomplete DMEM, for a total of in 50 μl for each well. After 45 min of complex formation, the 50 μl complex mixture was overlaid on the cell media (100 μl). At 7 h post-transfection, normalized luciferase activity (firefly/*Renilla* luciferase) was determined using Dual-Luciferase® Reporter (DLR™) assay system (Promega).

### *Quantitative RT-PCR*

LMH cells were co-transfected in 6-well plates with microRNA mimics and antagomiRs along with a plasmid expressing ICP4 (pRcICP4 (Fuchs et al., 2000)). Total RNA was extracted using Trizol (Invitrogen), analyzed with a 2100 Bioanalyzer (Agilent), and subjected to DNAse digestion prior to RT-PCR. qRT-PCR was performed using SYBR® GreenER™ master mix with Superscript III enzyme mix (Invitrogen). In control reactions, reverse transcriptase was omitted to confirm the absence of DNA in all samples. For some samples, a dilution series was performed to confirm the absence of PCR inhibitors in the RNA sample. The efficiency of amplification ranged from 80% to 85% as determined by the slope of the regression of RNA dilution versus threshold cycle values. The calculation of normalized target mRNA relative to the endogenous control (GAPDH) according to the delta-delta-CT was done according to the method described previously (Livak and Schmittgen, 2001). Using this method, efficiencies of both target and endogenous mRNA amplifications should be comparable, but need not be 100%, as long as the efficiencies of the two transcripts compared within the experiment are approximately equal. To test this, for each primer pair, we used the target and endogenous delta-$C_T$ data from the dilution series of the RNA samples, and all primer pairs met the criteria as outlined in this test.

All ICP4 RT-PCR reactions were normalized to chicken GAPDH, and relative quantities (RQ, $2^{-ddCT}$) were calculated (Livak and Schmittgen, 2001). Chicken GAPDH was quantified using GAPDH-F and GAPDH-R (Yang et al., 2007) with a final concentration of 0.05 μM. Two regions of ICP4 mRNA were amplified using primers described in Supplemental Table 1, with a final primer concentration of 0.05 μM. RQ values from ICP4 amplification were determined in samples treated with microRNA mimics and antagomiRs, and these RQ values were compared to treatments with 300 nM or 600 nM negative control mimics (Winer et al., 1999). Student's *t*-test (two-tailed) was performed to compare RQ of treatments to those of either 300 nM or 600 nM negative control mimic treatments.

### *Modified RACE*

LMH cells in four T75 flasks were infected with ILTV USDA strain, and RNA was harvested at 19 h post-infection. Total RNA was isolated using the RNeasy RNA miniprep kit (Qiagen, Inc.), and mRNA was isolated with the PolyATract® mRNA Isolation System (Promega). PolyA-selected RNA was subjected to RT-PCR using primers for cellular GAPDH (Yang et al., 2007) and ILTV glycoprotein C (Callison et al., 2007) with and without reverse transcriptase to assess DNA contamination. The ligation for the modified RACE used 1.2 μg mRNA in a 20 μl ligation reaction containing 15 U T4 RNA ligase (Ambion, Inc.) and the RNA oligonucleotide adapter (Dharmacon Research). The ligated RNA was purified using an YM30 ultracentrifugation unit (Amicon, Inc.), and 300 ng of the purified ligated RNA was used in first strand synthesis using primer 4079R1 (Supplemental Table 1) and Superscript II according to the manufacturer's instructions. Primary and secondary PCRs contained primers as outlined in Supplemental Table 1. The primary PCR (50 μl) contained 4 μl of the first strand reaction, and the secondary PCR (50 μl) contained 2–4 μl of the primary PCR. PCR products were cloned directly into pCR2.1-TOPO (Invitrogen) and sequenced using the M13 reverse primer.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.virol.2010.12.023.

## References

Bagust, T.J., Jones, R.C., Guy, J.S., 2000. Avian infectious laryngotracheitis. Revue Scientifique Et Technique De L Office International Des Epizooties 19 (2), 483–492.
Bartel, D.P., 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116 (2), 281–297.
Bartel, D.P., 2009. MicroRNAs: target recognition and regulatory functions. Cell 136 (2), 215–233.
Barth, S., Pfuhl, T., Mamiani, A., Ehses, C., Roemer, K., Kremmer, E., Jaker, C., Hock, J., Meister, G., Grasser, F.A., 2008. Epstein-Barr virus-encoded microRNA miR-BART2 down-regulates the viral DNA polymerase BALF5. Nucleic Acids Res. 36 (2), 666–675.
Block, T.M., Spivack, J.G., Steiner, I., Deshmane, S., McIntosh, M.T., Lirette, R.P., Fraser, N. W., 1990. A herpes simplex virus type 1 latency-associated transcript mutant reactivates with normal kinetics from latent infection. J. Virol. 64 (7), 3417–3426.
Burnside, J., Bernberg, E., Anderson, A., Lu, C., Meyers, B.C., Green, P.J., Jain, N., Isaacs, G., Morgan, R.W., 2006. Marek's disease virus encodes microRNAs that map to meq and the latency-associated transcript. J. Virol. 80 (17), 8778–8786.
Cai, X., Lu, S., Zhang, S., Gonzalez, C.M., Damania, B., Cullen, B.R., 2005. Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells. PNAS 102 (15), 5570–5575.
Callison, S.A., Riblet, S.M., Oldoni, I., Sun, S., Zavala, G., Williams, S., Resurreccion, R.S., Spackman, E., Garcia, M., 2007. Development and validation of a real-time Taqman PCR assay for the detection and quantitation of infectious laryngotracheitis virus in poultry. J. Virol. Meth. 139 (1), 31–38.
Cantello, J.L., Anderson, A.S., Morgan, R.W., 1994. Identification of latency-associated transcripts that map antisense to the ICP4 homolog gene of Marek's disease virus. J. Virol. 68 (10), 6280–6290.
Cantello, J.L., Parcells, M.S., Anderson, A.S., Morgan, R.W., 1997. Marek's disease virus latency-associated transcripts belong to a family of spliced RNAs that are antisense to the ICP4 homolog gene. J. Virol. 71 (2), 1353–1361.
Cullen, B.R., 2006. Viruses and microRNAs. Nat. Genet. 38, 525–530.

Cullen, B.R., 2009. Viral and cellular messenger RNA targets of viral microRNAs. Nature 457 (7228), 421–425.
Filipowicz, W., Bhattacharyya, S.N., Sonenberg, N., 2008. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat. Rev. Genet. 9 (2), 102–114.
Franco-Zorrilla, J.M., Valli, A., Todesco, M., Mateos, I., Puga, M.I., Rubio-Somoza, I., Leyva, A., Weigel, D., Garcia, J.A., Paz-Ares, J., 2007. Target mimicry provides a new mechanism for regulation of microRNA activity. Nat. Genet. 39 (8), 1033–1037.
Friedman, R.C., Farh, K.K.H., Burge, C.B., Bartel, D.P., 2009. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 19 (1), 92–105.
Fuchs, W., Ziemann, K., Teifke, J.P., Werner, O., Mettenleiter, T.C., 2000. The non-essential UL50 gene of avian infectious laryngotracheitis virus encodes a functional dUTPase which is not a virulence factor. J. Gen. Virol. 81, 627–638.
Garcia, M., Riblet, S.M., 2001. Characterization of infectious laryngotracheitis virus isolates: demonstration of viral subpopulations within vaccine preparations. Avian Dis. 45 (3), 558–566.
German, M.A., Pillay, M., Jeong, D.H., Hetawal, A., Luo, S.J., Janardhanan, P., Kannan, V., Rymarquis, L.A., Nobuta, K., German, R., De Paoli, E., Lu, C., Schroth, G., Meyers, B.C., Green, P.J., 2008. Global identification of microRNA-target RNA pairs by parallel analysis of RNA ends. Nat. Biotechnol. 26 (8), 941–946.
Gluzman, Y., 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell 23 (1), 175–182.
Grey, F., Antoniewicz, A., Allen, E., Saugstad, J., McShea, A., Carrington, J.C., Nelson, J., 2005. Identification and characterization of human cytomegalovirus-encoded microRNAs. J. Virol. 79 (18), 12095–12099.
Grey, F., Meyers, H., White, E.A., Spector, D.H., Nelson, J., 2007. A human cytomegalovirus-encoded microRNA regulates expression of multiple viral genes involved in replication. PLoS Path. 3 (11), 1593–1602.
Grey, F., Hook, L., Nelson, J., 2008. The functions of herpesvirus-encoded microRNAs. Med. Microbiol. Immunol. 197 (2), 261–267.
Grundhoff, A., Sullivan, C.S., Ganem, D., 2006. A combined computational and microarray-based approach identifies novel microRNAs encoded by human gamma-herpesviruses. RNA 12 (5), 733–750.
He, L., Hannon, G.J., 2004. MicroRNAs: small RNAs with a big role in gene regulation. Nat. Rev. Genet. 5 (7), 522–531.
Ho, D.Y., Mocarski, E.S., 1988. Beta-galactosidase as a marker in the peripheral and neural tissues of the herpes simplex virus-infected mouse. Virology 167 (1), 279–283.
Hobert, O., 2008. Gene regulation by transcription factors and microRNAs. Science 319 (5871), 1785–1786.
Hughes, C.S., Gaskell, R.M., Jones, R.C., Bradbury, J.M., Jordan, F.T.W., 1989. Effects of certain stress factors on the re-excretion of infectious laryngotracheitis virus from latently infected carrier birds. Res. Vet. Sci. 46 (2), 274–276.
Hughes, C.S., Williams, R.A., Gaskell, R.M., Jordan, F.T.W., Bradbury, J.M., Bennett, M., Jones, R.C., 1991. Latency and reactivation of infectious laryngotracheitis vaccine virus. Arch. Virol. 121 (1–4), 213–218.
Johnson, M.A., Tyack, S.G., Prideaux, C., Kongsuwan, K., Sheppard, M., 1995. Nucleotide sequence of infectious laryngotracheitis virus (Gallidherpesvirus-1) ICP4 gene. Virus Res. 35 (2), 193–204.
Jurak, I., Kramer, M.F., Mellor, J.C., van Lint, A.L., Roth, F.P., Knipe, D.M., Coen, D.M., 2010. Numerous conserved and divergent microRNAs expressed by herpes simplex viruses 1 and 2. J. Virol. 84 (9), 4659–4672.
Kang, W., Mukerjee, R., Fraser, N.W., 2003. Establishment and maintenance of HSV latent infection is mediated through correct splicing of the LAT primary transcript. Virology 312 (1), 233–244.
Kawaguchi, T., Nomura, K., Hirayama, Y., Kitagawa, T., 1987. Establishment and characterization of a chicken hepatocellular carcinoma cell line, LMH. Cancer Res. 47, 4460–4464.
Kirkpatrick, N.C., Mahmoudian, A., Colson, C.A., Devlin, J.M., Noormohammadi, A.H., 2006. Relationship between mortality, clinical signs and tracheal pathology in infectious laryngotracheitis. Avian Pathol. 35 (6), 449–453.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25 (4), 402–408.
Llave, C., Xie, Z.X., Kasschau, K.D., Carrington, J.C., 2002. Cleavage of Scarecrow-like mRNA targets directed by a class of *Arabidopsis* miRNA. Science 297 (5589), 2053–2056.
Long, D., Lee, R., Williams, P., Chan, C.Y., Ambros, V., Ding, Y., 2007. Potent effect of target structure on microRNA function. Nat. Struct. Mol. Biol. 14 (4), 287–294.
Morgan, R.W., Xie, Q., Cantello, J.L., Miles, A.M., Bernberg, E.L., Kent, J., Anderson, A., 2001. Marek's disease virus latency. In: K., Hirai (Ed.), Marek's Disease. Springer-Verlag, Berlin, pp. 223–243.
Murphy, E., Vanicek, J., Robins, H., Shenk, T., Levine, A.J., 2008. Suppression of immediate-early viral gene expression by herpesvirus-coded microRNAs: implications for latency. PNAS 105 (14), 5453–5458.
Nilsen, T.W., 2007. Mechanisms of microRNA-mediated gene regulation in animal cells. Trends Genet. 23 (5), 243–249.
Obernosterer, G., Tafer, H., Martinez, J., 2008. Target site effects in the RNA interference and microRNA pathways. Biochem. Soc. Trans. 36, 1216–1219.
Perng, G.C., Jones, C., Ciacci-Zanella, J., Stone, M., Henderson, G., Yukht, A., Slanina, S.M., Hofman, F.M., Ghiasi, H., Nesburn, A.B., Wechsler, S.L., 2000. Virus-induced neuronal apoptosis blocked by the herpes simplex virus latency-associated transcript. Science 287 (5457), 1500–1503.
Pfeffer, S., Zavolan, M., Grasser, F.A., Chien, M., Russo, J.J., Ju, J., John, B., Enright, A.J., Marks, D., Sander, C., Tuschl, T., 2004. Identification of virus-encoded microRNAs. Science 304, 734–736.
Pfeffer, S., Sewer, A., Lagos-Quintana, M., Sheridan, R., Sander, C., Grasser, F.A., vanDyk, L.F., Ho, C.K., Shuman, S., Chien, M., Russo, J.J., Ju, J., Randall, G., Lindenbach, B.D., Rice, C.M., Simon, V., Ho, D.D., Zavolan, M., Tuschl, T., 2005. Identification of microRNAs of the herpesvirus family. Nat. Meth. 2 (4), 269–276.
Rachamadugu, R., Lee, J.Y., Wooming, A., Kong, B.W., 2009. Identification and expression analysis of infectious laryngotracheitis virus encoding microRNAs. Virus Genes 39 (3), 301–308.
Samols, M.A., Hu, J., Skalsky, R.L., Renne, R., 2005. Cloning and identification of a microRNA cluster within the latency-associated region of Kaposi's sarcoma-associated herpesvirus. J. Virol. 79 (14), 9301–9305.
Sawtell, N.M., 1997. Comprehensive quantification of herpes simplex virus latency at the single-cell level. J. Virol. 71 (7), 5423–5431.
Schafer, A., Cai, X.Z., Bilello, J.P., Desrosiers, R.C., Cullen, B.R., 2007. Cloning and analysis of microRNAs encoded by the primate gamma-herpesvirus rhesus monkey rhadinovirus. Virology 364 (1), 21–27.
Sedarati, F., Izumi, K.M., Wagner, E.K., Stevens, J.G., 1989. Herpes simplex virus type 1 latency-associated transcription plays no role in establishment or maintenance of a latent infection in murine sensory neurons. J. Virol. 63 (10), 4455–4458.
Sempere, L.F., Freemantle, S., Pitha-Rowe, I., Moss, E., Dmitrovsky, E., Ambros, V., 2004. Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. Genome Biol. 5 (3), R13.
Seo, G.J., Fink, L.H.L., O'Hara, B., Atwood, W.J., Sullivan, C.S., 2008. Evolutionarily conserved function of a viral microRNA. J. Virol. 82 (20), 9823–9828.
Seo, G.J., Chen, C.J., Sullivan, C.S., 2009. Merkel cell polyomavirus encodes a microRNA with the ability to autoregulate viral gene expression. Virology 383 (2), 183–187.
Stern-Ginossar, N., Saleh, N., Goldberg, M.D., Prichard, M., Wolf, D.G., Mandelboim, O., 2009. Analysis of human cytomegalovirus-encoded microRNA activity during infection. J. Virol. 83 (20), 10684–10693.
Sullivan, C.S., Grundhoff, A.T., Tevethia, S., Pipas, J.M., Ganem, D., 2005. SV40-encoded microRNAs regulate viral gene expression and reduce susceptibility to cytotoxic T cells. Nature 435 (7042), 682–686.
Sullivan, C.S., Sung, C.K., Pack, C.D., Grundhoff, A., Lukacher, A.E., Benjamin, T.L., Ganem, D., 2009. Murine Polyomavirus encodes a microRNA that cleaves early RNA transcripts but is not essential for experimental infection. Virology 387 (1), 157–167.
Tang, S., Bertke, A.S., Patel, A., Wang, K., Cohen, J.I., Krause, P.R., 2008. An acutely and latently expressed herpes simplex virus 2 viral microRNA inhibits expression of ICP34.5, a viral neurovirulence factor. PNAS 105 (31), 10931–10936.
Tang, S., Patel, A., Krause, P.R., 2009. Novel less-abundant viral microRNAs encoded by herpes simplex virus 2 latency-associated transcript and their roles in regulating ICP34.5 and ICP0 mRNAs. J. Virol. 83 (3), 1433–1442.
Umbach, J.L., Kramer, M.F., Jurak, I., Karnowski, H.W., Coen, D.M., Cullen, B.R., 2008. MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. Nature 454 (7205), 780–783.
Waidner, L.A., Morgan, R.W., Anderson, A.S., Bernberg, E.L., Kamboj, S., Garcia, M., Riblet, S.M., Ouyang, M., Isaacs, G.K., Markis, M., Meyers, B.C., Green, P.J., Burnside, J., 2009. MicroRNAs of Gallid and Meleagrid herpesviruses show generally conserved genomic locations and are virus-specific. Virology 388 (1), 128–136.
Wang, X.J., Reyes, J.L., Chua, N.H., Gaasterland, T., 2004. Prediction and identification of Arabidopsis thaliana microRNAs and their mRNA targets. Genome Biol. 5 (9), R65.
Williams, R.A., Bennett, M., Bradbury, J.M., Gaskell, R.M., Jones, R.C., Jordan, F.T.W., 1992. Demonstration of sites of latency of infectious laryngotracheitis virus using the polymerase chain-reaction. J. Gen. Virol. 73, 2415–2420.
Winer, J., Jung, C.K.S., Shackel, I., Williams, P.M., 1999. Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. Anal. Biochem. 270 (1), 41–49.
Yang, F., Xian, R.R., Li, Y.Y., Polony, T.S., Beemon, K.L., 2007. Telornerase reverse transcriptase expression elevated by avian leukosis virus integration in B cell lymphomas. PNAS 104 (48), 18952–18957.
Yao, Y., Zhao, Y., Xu, H., Smith, L.P., Lawrie, C.H., Sewer, A., Zavolan, M., Nair, V., 2007. Marek's disease virus type 2 (MDV-2)-encoded microRNAs show no sequence conservation with those encoded by MDV-1. J. Virol. 81 (13), 7164–7170.
Yao, Y., Zhao, Y., Xu, H., Smith, L.P., Lawrie, C.H., Watson, M., Nair, V., 2008. MicroRNA profile of Marek's disease virus-transformed T-cell line MSB-1: predominance of virus-encoded microRNAs. J. Virol. 82 (8), 4007–4015.
Yekta, S., Shih, I.-H., Bartel, D.P., 2004. MicroRNA-directed ceavage of HOXB8 mRNA. Science 304 (5670), 594–596.