MicroRNAs derived from the insect virus HzNV-1 promote lytic infection by suppressing histone methylation

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Heliothis zea nudivirus-1 (HzNV-1) is an insect virus that can induce both lytic and latent infections in various insect cell lines. During latent infection, several microRNAs (miRNAs) are produced from persistency-associated gene 1 (pag1) as the only detectable HzNV-1 transcript. Previous studies have shown that the pag1 gene suppresses the immediate-early gene hhi1 and promotes host switching into a latent infection via miRNAs derived from pag1. Although other functions of the miRNAs derived from pag1 have not yet been elucidated, several studies have suggested that miRNAs encoded from latency-associated genes can regulate histone-associated enzymes. Because pag1 is a noncoding transcript, it potentially regulates host chromatin structure through miRNAs upon infection. Nevertheless, the exact mechanism by which pag1 alters viral infections remains unknown. In this study, we found that the pag1-encoded miRNA miR-420 suppresses expression of the histone modification-associated enzyme su(var)3–9. Therefore, this miRNA causes histone modification to promote HzNV-1 infection. These results suggest that HzNV-1 may directly influence epigenetic regulation in host cells through interactions with pag1 miRNAs to promote lytic infection. This study provides us with a better understanding of both the HzNV-1 infection pathway and the relationship between viral miRNAs and epigenetic regulation.
Heliothis virescens ascovirus (HvAV)\(^1\) and West Nile virus (WNV), have also been observed to employ their own miRNAs to regulate viral replication\(^1\)\(^7\).

Epigenetic regulation involves histone modification\(^1\)\(^8\), DNA methylation/acetylation\(^1\)\(^9\), and miRNA expression\(^2\)\(^0\). Recently, Kaposi’s sarcoma-associated herpesvirus (KSHV) has been shown to regulate its lytic/latent cycle through histone modification\(^2\)\(^1\), and active or repressive histone markers are reportedly distributed across the entire latent KSHV genome, suggesting that this phenomenon is dynamic\(^2\)\(^2\)\(^,\)\(^2\)\(^3\). Histone H3-lysine\(^2\)\(^4\) trimethylation (H3K27me3), the dominant repressive marker, covers the majority of the KSHV genome and suppresses lytic gene expression. In addition to KSHV, almost all human herpesviruses (HSV, KHSV and HCMV) encode viral miRNAs that inhibit IE gene expression to regulate lytic and latent infection\(^2\)\(^5\)\(^,\)\(^2\)\(^6\). Methylation and acetylation of histone tails are carried out by DNA methyltransferases (DNMTs) and histone acetyltransferases (HATs), respectively\(^2\)\(^4\)\(^,\)\(^2\)\(^7\). There are two forms of chromatin: heterochromatin and euchromatin. The former is defined as condensed chromatin in which transcription is blocked, whereas euchromatin is defined as lightly packed, unstable chromatin that is often present during active transcription\(^1\)\(^8\)\(^,\)\(^2\)\(^8\). Histone-lysine N-methyltransferase Su(var) function is related to heterochromatin with position-effect variegation (PEV)\(^3\)\(^9\)\(^,\)\(^3\)\(^0\), and one member of the su(var) group, su(var)3–9, that causes histone H3-lysine\(^3\) (H3K9) methylation has been extensively studied in Drosophila and mammals\(^3\)\(^1\)\(^,\)\(^3\)\(^2\). Furthermore, su(var) is present in Spodoptera frugiperda and two additional Spodoptera species and is associated with the silencing of viral gene expression through interactions with epigenetic factors\(^3\)\(^3\). Another gene, tip60, encoding a HAT is also associated with pathogenic infection in Lepidoptera insects\(^3\)\(^4\).

HSV's have been found to modify host histone tails during virus infection. As these reports primarily focused on the promoters of lytic and latent genes and assessed histone methylation associated with gene suppression\(^3\)\(^5\), sites with higher levels of methylation during the latent stage have been well studied\(^3\)\(^5\)\(^,\)\(^3\)\(^6\). In contrast, studies of epigenetic regulation in viruses are still limited. Therefore, we used the pag1 gene, which produces the only detectable gene transcript during latent HzNV-1 infection, as a model system to study whether pag1-encoded miRNAs directly affect host histone modification. The results of this study suggest that this is not the case. We chose HzNV-1, which has features similar to those of HSV's, as our model system. We first observed a decline in H3K9me3 levels, in contrast to other reports, whereas acetylation levels were significantly enhanced by the presence of the pag1 gene. We next identified two histone transferases that might be responsible for these phenomena, su(var)3–9 and tip60. We demonstrated that su(var)3–9 was downregulated by a miRNA encoded from pag1: miR-420. We found evidence supporting that HzNV-1 uses its latent infection-associated miRNAs to control host histone modification and promote lytic infection. We propose a model that accounts for the latent infection of insect viruses and can also be applied to mammalian viruses.

Results

Histone modification detected at different time points after pag1 transfection. As previous studies have shown that pag1 regulated viral gene expression and influenced the type of infection\(^3\)\(^7\), the association between the pag1 gene and host histone status was therefore examined. Since the amino-terminal tail of histone 3 has been studied thoroughly in recent years, the histone 3 modification pattern was chosen as a reference for examining changes in histone modifications caused by pag1 using acetylation H3 and trimethylated H3K9 antibodies (Fig. 1). After pag1 was transfected into cells, strong acetylation was observed up to

![Figure 1.](image-url)
The acetylation levels of pag1 samples were higher than those of the control group throughout the experiment and peaked at 24 hours post-transfection (Fig. 1A); conversely, methylation in host cells decreased after transfection (Fig. 1B). These data suggest that the pag1 gene may encode factor(s), which would upregulate gene expression. Although previous studies have shown that methylation levels of lytic gene transcripts may increase during latent infection, the distribution of epigenetic markers is suggested to be dynamic across the entire host genome during the latent stage.

To further confirm that pag1 was able to alter host histone modifications, two histone-associated enzymes, HATs tip60 and su(var)3–9, were chosen to explore the role of the pag1 gene in the histone modification pathway. The expression levels of HAT tip60 and su(var)3–9 at 0 to 48 hours post pag1 transfection were measured. Previous studies have revealed that HAT tip60 is associated with H3 acetylation, and the result here showed that tip60 expression was increased upon pag1 transfection (Fig. 2B), which was in agreement with the western blot results (Fig. 1B) and previous findings, suggesting that acetylation increased after pag1 transfection. The acetylation levels after pag1 transfection were 3-fold higher than those in the control group at 48 hours post-transfection (Fig. 2A,B), and the presence of pag1 suppressed su(var)3–9 expression by 50% (Fig. 2C,D). It is worth noting that pag1 does not encode a protein, but instead miRNAs are produced. Several studies have suggested that the miRNAs produced from latent-associated genes are mostly involved in regulating histone-associated enzymes36. Su(var)3–9 trimethylates H3K9, and it has been identified in different animals33, though similar mechanisms in insects have not yet been identified. Therefore, miRNAs may be crucial for the mechanism by which HzNV-1 affects these two histone transferases.

**Predicted and identified miRNAs produced by the pag1 transcript.** According to the NCBI database, the complete genome of HzNV-1 is approximately 228 kb, and the pag1 transcript is approximately 4 kb. To identify miRNAs that target the histone transferases encoded by tip60 and su(var)3–9, pag1-encoded miRNAs were mapped onto these genes. Prediction was performed by Vir-Mir and based on mapping scores and structures. Four candidate miRNAs (Hv-miR-420, Hv-miR-795, Hv-miR-150, Hv-miR-475) were selected for further evaluation (Fig. 3). The sequences of the miRNAs are listed in Table 1. To verify that the predicted miRNAs are actually encoded and generated from pag1, stem-loop RT-qPCR was used to measure these candidate miRNAs at different time points after pag1 transfection in Sf-21 cells. Among these miRNAs, Hv-miR-795 (Fig. 4A) and Hv-miR-475 (Fig. 4B) were detected in both pag1-transfected cells and in untransfected cells, suggesting that host cells also expressed these 2 miRNAs. Hv-miR-150 was undetectable in both groups, indicating that Hv-miR-150 is a mock miRNA (Fig. 4C). Hv-miR-420 was the only candidate miRNA whose expression was significantly increased after pag1 transfection (Fig. 4D). To further confirm the results of stem-loop PCR, northern blot analysis was performed, showing detection of Hv-miR-420 in both pag1-transfected and HzNV-1-infected...
Hv-miR-420 suppresses su(var)3–9 expression. Although the functions of several pag1 miRNAs have been identified, the remaining miRNAs predicted from the pag1 transcript need to be further studied. To address this, the miRNAs predicted from pag1 were mapped onto the tip60 and su(var)3–9 sequences (Fig. 3) and, based on the stem-loop PCR assay and the mode of action of miRNAs in general, Hv-miR-420 was selected for further experiments (Fig. 4). To evaluate the effect of miRNA-420 on su(var)3–9, Hv-miR-420 mimic was transfected into Sf-21 cells and it was found that 60% of su(var)3–9 expression was suppressed 72 hours after transfection (Fig. 5A). To prove a direct relationship between Hv-miR-420 and suppression of su(var)3–9, Hv-miR-420 inhibitor was transfected into pag1-transfected cells (pag1 transfected 24 hours prior to inhibitor transfection). Notably, the levels of su(var)3–9 expression recovered to approximately 72% of the untreated control in contrast to 50% of the expression level in the pag1-transfected cells without inhibitor treatment (Fig. 5B). Hv-miR-420 expression levels in HzNV-1-infected cells was measured and it was found to significantly increased after viral infection (Fig. 5C), where su(var)3–9 protein levels was significantly decreased (Fig. 5D). Moreover, transfection of the Hv-miR-420 inhibitor increased Su(var)3–9 protein levels compared to HzNV-1-infected cells without inhibitor (Fig. 5D). To confirm direct interaction between miR-420 and su(var)3–9, a mutant miR-420 which contained base mutations in its seed region was generated (designated miR-420m, Fig. 6A). Western blotting analysis (Fig. 6B) showed that the mutant miR420 did not affect the levels of Su(var)3–9 expression, suggesting that intact seed sequence was crucial for functioning. To assess whether the observed regulation was direct, the 3’ UTR of su(var)3–9 was fused to the 3’-end of the egfp gene to generate an EGFP reporter plasmid. Co-transfection of this EGFP reporter plasmid with Hv-miR-420 significantly decreased EGFP levels, whereas co-transfection with the Hv-miR-420m did not affect EGFP expression (Fig. 6C). These results demonstrate that su(var)3–9 expression was suppressed by increased Hv-miR-420 levels in both pag1-transfected and virus-infected cells.

Hv-miR-420 is involved in regulating HzNV-1 lytic infection. Previous studies have found that the pag1 transcript is the only HzNV-1 viral transcript expressed during both latent and lytic infections. In this study, changes in Hv-miR-420 expression at different infection stages were examined to evaluate whether Hv-miR-420 affected histone modification and altered infection stages. Virus titers in both latent infection cell lines (SFP4) and HzNV-1-infected Sf-21 cells were analyzed following reactivation from latent stage induced by transfection of hhi1 expression plasmid (Fig. 7A). Transfection of hhi1 into HzNV-1-latently-infected SFP4 cells significantly increased Hv-miR-420 expression (Fig. 7B) but the expression of su(var)3–9 was inhibited (Fig. 7C), indicating that Hv-miR-420 played essential roles in altering histone modification by latent viruses after reactivation.
assess whether Hv-miR-420 was able to stimulate latent viruses to enter lytic infection stage, Hv-miR-420 mimic was transfected into SFP4 cells and analyzed viral reactivation. The results showed significant cell lysis within 72 hours and increased virus titers after Hv-miR-420 transfection (Fig. 7D). These results demonstrated that Hv-miR-420 was able to induce latent viruses to enter lytic infection stage, possibly by contributing to activation of viral gene expression through inhibition of histone methylation.

Discussion

The characteristics of HzNV-1 are universal among mammalian viruses, especially HSVs, which share several common features with HzNV-1. The noncoding latency-associated transcript (LAT) gene is highly expressed during HSV or HCMV latency and encodes a series of miRNAs. Previous studies have focused on the interaction between viral miRNAs and viral genes, and it has been reported that LAT-encoded miRNAs not only inhibit the HSV IE genes ICP0 and ICP4 but also regulate histone modification. However, these studies are still limited in insect viruses.

In this study, we discovered that the latent gene of HzNV-1, pag1, was able to affect two insect histone transferases, tip60 and su(var)3–9, at the beginning phase of infection (Fig. 2). tip60 is involved in a variety of processes, such as DNA repair, development, and human virus association. su(var)3–9, on the other hand, is a conserved histone transferase that also possesses multiple roles in animals and is related to the heterochromatin-associated HP1 protein together with H3K9 trimethylation. We examined the expression of histone proteins containing modification in regions that are targets of the abovementioned histone transferases. Western blot analysis showed that trimethylation of H3K9 was significantly downregulated and that acetylation of H3 was upregulated after pag1 transfection (Fig. 1). A possible explanation is that when methyltransferase expression decreases, resulting in reduced trimethylation of histones (H3K9), more histones are available for acetyl modification, leading to an increase in acetylated H3.

Acetylation of histones relaxes the structure of chromatin, typically resulting in gene activation. In contrast, methylation is reported to be associated with heterochromatin and gene silencing. For this reason, most studies are inclined to relate methylation and deacetylation with latent infection. However, the ICP0 gene of HSV was found to be acetylated during quiescence. Additionally, three early genes of KSHV are reported to have distinct histone modifications after KSHV primary infection. Indeed, relieving HDAC repression leads to activation of

![Figure 4](https://www.nature.com/scientificreports/) Stem-loop RT-qPCR confirmation of miRNA expression after pag1 transfection. (A) Hv-miR-795 levels decreased after pag1 transfection. (B) Hv-miR-475 was not affected by pag1 in Sf-21 cells. (C) Hv-miR-150 was not detected at any time point. (D) Hv-miR-420 was identified after pag1 transfection. (E) Small RNAs harvested from pag1-transfected cells and HzNV-1 productively infected cells were analyzed by northern blotting using probes against predicted HzNV-1 miR-420 (top panels) or let-7a miRNA as a positive control (bottom panels). At least three repetitions were conducted for each group. P values were calculated using Student’s t-test (*P < 0.05).
lytic genes in both HCMV and EBV. This study indicates that repressive and active markers are simultaneously distributed across chromatin during latency.

Similar to the LAT gene of HSV, pag1 plays an important role during HzNV-1 latent infection. Previous studies have shown that the pag1 transcript yields several miRNAs, some of which were shown to maintain latency. The ability of miR-420 to stimulate a virus toward lytic infection may be due to differential expression of pag1-derived miRNAs during lytic and latent stages. Our preliminary results show relatively low miR-420 expression in latently infected cells, suggesting that the generation of miR-420 was downregulated. Investigation of how the expression of pag1-derived microRNAs are regulated is currently underway.

The difficulty in establishing a stable HSV-latently-infected cell line has hampered the progress of HSV latent infection research. Nonetheless, a permanent cell line of HzNV-1 latent infection, namely, SFP4 cells, is available and the results of pag1 transfection could be validated in the context of HzNV-1 latent infection. Indeed, we performed experiments with pag1 to eliminate other variables correlated with HzNV-1. Our discovery of an epigenetic pathway that had been modified by Hv-miR-420 indicates that some variables regarding Hv-miR-420 and HMT su(var)3–9 in the dual stages of HzNV-1 may need to be taken into consideration in the future.
to an earlier study, pag1 is detectable in both lytic and latent stages. Under these circumstances, we may be able to utilize a reactivating virus from SFP4 cells to compare different expression levels of Hv-miR-420, su(var)3−9, and trimethylation H3K9, which can help in determining whether this microRNA has a crucial role in reactivating HzNV-1.

Previous studies on human latent viruses, e.g., HSV50, KSHV 21, HBV 51 and HCMV 52, found that host HATs are affected by these viruses. In contrast, few studies have investigated interaction between viral miRNAs and histone modification. Our research presented herein addressed this interaction and revealed that Hv-miR420, a miRNA produced from the pag1 gene, regulates host HMT su(var)3−9 to suppress H3K9 trimethylation. Surprisingly, a tentative explanation is that the decrease in H3K9 trimethylation during latency is a sign of reactivation (Fig. 8).

The physiological significance and in vitro tests need to be pursued to further support and verify our findings. This work on the functions of latent miRNAs from an epigenetic perspective provides a better understanding of insect latent viruses.

Materials and Methods

Cell culture and virus. S. frugiperda IPLB-Sf-21 cells were cultured in TC-100 insect cell medium with 10% fetal bovine serum (FBS) at 26 °C (Gibco BRL) 53. The SFP4 cell line was derived from latently infected Sf-21 cells 4, 5. HzNV-1 titers were calculated using TCID 50 54 and quantitative PCR (qPCR) 55.

Plasmid DNA construction and transfection. The coding regions of hhil and pag1 were previously inserted into the vector pKSh to construct pKShH1 and pKShP1 1. To clone the 3′-UTR of su(var)3−9 (primer: 3′-9 3UTR-F: ACGCACGCTCATTCTGACACACGC; 3′-9 3UTR-R: TTTACAATCTTATTACATTTAC) into an EGFP reporter vector (pKShE) 5, the su(var)3−9-UTR fragment was inserted into the EcoRI site after the stop codon of the EGFP gene (pKShEUT). A total of 2 × 10^6 Sf-21 cells were seeded into a 24-well culture plate (Corning, Action, MA) and transfected with 0.5 μg of plasmid DNA using Cellfectin (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol (Gibco BRL). EGFP expression was observed using fluorescent microscope at 2 days-post transfection. Samples were collected at 48 hours post-transfection in triplicate.
Western blotting. Proteins were isolated with 40 μl of RIPA buffer (Thermo Fisher) per well in a 24-well culture plate, and the samples were immediately stored at −80 °C until use. Western blotting was carried out using rabbit polyclonal antibodies detecting acetyl-H3 (Millipore, 1:4500), trimethyl-H3K9 (Millipore, 1:500), su(var)3–9 (Abcam, 1:4000) and EGFP (Millipore, 1:5000). Normalization was performed via detection of actin levels using a mouse polyclonal antibody (Millipore, 1:2500). All bands were analyzed and quantified using AlphaView SA.

RT-PCR and qPCR. RNA samples were collected using 100 μl of GENEzol™ reagent per cm² of culture dish with a GENEzol™ TriRNA Pure Kit (Geneaid) and stored at −20 °C. The eluted RNA was reverse transcribed using a PrimeScript™ RT-PCR Kit (TaKaRa), and cDNA was synthesized from 800 ng of RNA for each sample. Quantification of gene expression was carried out by adding 2 μl of cDNA to 2 × SeniFAST SYBR® Hi-ROX Mix (BIOLINE) using a 56 °C annealing temperature. PCR was carried out using the following primers:

- **Actin** F: TCAACCCCAAGGCCAACAGAGA
- **Actin** R: GAGGCTCCTCGGAATTTTC
- **Tip60** F: CCGGAAATGGGAATACACAG
- **Tip60** R: TGGAGAGCCACATAACAACCTG
- **Su(var)3–9** F: CCGCTGTCGGACTCAGTTAT
- **Su(var)3–9** R: GAGGCTCCTCGGAATTTTC

Computational prediction of viral miRNAs. *pag1* miRNA prediction was implemented according to the full-length gene sequence of *pag1* (NC_004156.1), which was obtained from the National Center for Biotechnology Information. The prediction was conducted using the miRNA prediction database (Vir-Mir) ([http://alk.ibms.sinica.edu.tw](http://alk.ibms.sinica.edu.tw)). The miRNAs with high match scores chosen for analysis are listed in Table 1.

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**Figure 7.** Induction of latent viral reactivation by miR-420. (A) Viral titers estimated in productively and latently infected cells. Latently infected cells were transfected with the plasmid pKShH1. (B) Stem-loop RT-qPCR confirmed miR-420 expression in *hhi1*-transfected SFP4 cells or *HzNV*-infected Sf-21 cells. (C) Su(var)3–9 expression was downregulated in *hhi1*-transfected SFP4 cells. (D) Viral titers were estimated in latently infected cells. Latently infected cells were transfected with miR-420 mimic and control miRNA, and viral titers were determined at 72 hours post-transfection. Sf-21 cells were infected with the wild-type virus as a control. At least three repetitions were conducted for each group. P values were calculated using Student’s t-test (*P < 0.05).
Stem-loop real-time PCR and northern blotting. Sf-21 cells transfected with pKShP1 at 0 and 48 hpt were used for extraction of total RNA using TRIzol® reagent (Invitrogen) following the manufacturer’s instructions. The method for detecting mature miRNAs with specific primers was described in a previous study. The miRNA primers used for RT-PCR and real-time qPCR were as follows:

- Hv-miR-420 RT: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTG GATACGACTGTAT-3′
- Hv-miR-420 F: 5′-CACGCATTGGCAATTATA-3′
- Hv-miR-150 RT: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTG GATACGACCTTTGA-3′
- Hv-miR-150 F: 5′-CACGCAATCCATGATTTAA-3′
- Hv-miR-795 RT: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTG GATACGACCCGGA-3′
- Hv-miR-795 F: 5′-CACGCAGTCTTTTGTAGTATTACG-3′
- Hv-miR-475 RT: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTG GATACGACACCTTTA-3′
- Hv-miR-475 F: 5′-CACGCACACCACACCATTTCG-3′
- Reverse primer: 5′-CCAGTGGCAGGTCGTCGAGTA-3′

Total small RNA samples from pag1-transfected and HeNV-1-infected cells were isolated using a mirVanaTM miRNA isolation kit (Ambion) according to the manufacturer’s instructions. For northern blot analysis of small RNAs, 1 μg of small RNA sample and radio-labeled Decade Marker (Ambion) were fractionated by 15% denaturing polyacrylamide gel electrophoresis (PAGE) (acrylamide:bis ratio, 19:1) containing 8 M urea in 0.5 × TBE buffer. RNAs were transferred by electroblotting to a Hybond-N+ nylon transfer membrane (GE Healthcare) and UV cross-linked. RNA oligonucleotides (Integrated DNA Technologies) carrying the reverse complementary sequence for candidate miRNAs or let-7a were end-labeled with DIG (MP Biomedicals, Irvine, CA) to high

Figure 8. A model of the establishment of lytic infection via miR-420-mediated suppression of histone methylation. (A) Without miR-420. (B) With miR-420.
specific activity. Hybridizations and washes were carried out using DIG hybridization buffer according to the manufacturer's directions (Roche).

**Mimic and inhibitor miRNA transfection.** The Hv-miR-420 mimic was synthesized by the MDBio Incorporation, and inhibitor was produced by GenePharma Incorporation. The mimic (50 nM) or inhibitor was transfected into Si-21 cells using 1.5 μl of Lipofectamine™ RNAiMAX following the manufacturer's protocol (Invitrogen). The sequences of the Hv-miR-420 mimic, Hv-miR-420m and inhibitor are 5′-UUUGGCCAAYUUAUUAACCA-3′; 5′-UUUGACAGUUUAAUUAACCA-3′ and 5′-UUGUAUAAUAAAUUGGGCA-3′, respectively.

**Statistical analysis.** The qPCR Ct values for samples treated with plasmids were normalized to those of actin with the comparative Ct method\(^8\). Each group of experimental data was selected for comparison with the control groups using a single tail and type 1 t-test. The statistics used in this data analysis were performed with the T-TEST function in Microsoft Excel. P values are indicated in figures, with (*) representing the level of significance.

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### Additional Information

#### Competing Interests: The authors declare no competing interests.

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