JOURNAL OF VIROLOGY, Sept. 2008, p. 9164–9170 Vol. 82, No. 18
0022-538X/08/$08.00+0 doi:10.1128/JVI.01109-08
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An Insect Virus-Encoded MicroRNA Regulates Viral Replication▽

Mazhar Hussain,[1] Ryan J. Taft,[2] and Sassan Asgari[1]*

School of Integrative Biology, University of Queensland, St. Lucia, Queensland 4072, Australia,[1] and Institute for Molecular Bioscience, University of Queensland, St. Lucia, Queensland 4072, Australia[2]

Received 26 May 2008/Accepted 30 June 2008

MicroRNAs (miRNAs) are small (~22 nucleotides) noncoding RNAs which play an essential role in gene regulation and affect a wide range of processes, including development, differentiation, and oncogenesis. Here we report the identification of the first miRNA from an insect virus, derived from the major capsid protein (MCP) gene in *Heliothis virescens* ascovirus (HvAV) (HvAV-miR-1). Although MCP was abundantly expressed at all time points 24 h after infection, HvAV-miR-1 expression was strictly regulated and specifically detected from 96 h postinfection. HvAV-miR-1 expression coincided with a marked reduction of the expression of HvAV DNA polymerase I, which is a predicted target. Ectopic expression of full-length and truncated versions of MCP retaining the miRNA sequence significantly reduced DNA polymerase I transcript levels and inhibited viral replication. Our results indicate that HvAV-miR-1 directs transcriptional degradation of DNA polymerase I and regulates HvAV replication. These findings are congruent with recent reports that miR-BART-2 regulates Epstein-Barr virus DNA polymerase expression and suggest that virus-encoded miRNA regulation of virus replication may be a general phenomenon.

A tremendous amount of literature has been published in the last decade demonstrating that microRNAs (miRNAs) are fundamental regulators of gene expression and are involved in biological processes ranging from differentiation to development and immunological defense (36). miRNAs are 19- to 24-nucleotide (nt), noncoding small RNA molecules derived from RNA transcripts produced by both RNA polymerase II (2) and III (33). The primary miRNA transcript (pri-miRNA), which can be >2 kb, is cleaved by Drosha, yielding a 60- to 80-nt hairpin stem-loop pre-miRNA. The pre-miRNA is subsequently shuttled by exportins to the cytoplasm, where it is cleaved by Dicer into a duplex, one strand of which is incorporated into the RNA-induced silencing complex which targets an mRNA for degradation or translation repression, depending on the complementarity between the miRNA and the target (34).

With the help of advancements in bioinformatics, the development of new cloning techniques, and the introduction of deep sequencing, the number of miRNAs identified in plants, animals, and viruses is accelerating. Indeed, studies of virus-encoded miRNAs, predominantly from the double-stranded DNA *Herpesviridae* family, have shown that virus-encoded miRNAs target viral and host transcripts, facilitate host immune response evasion, and share targets with endogenous host miRNAs. For example, two recent studies have shown that a Kaposi's sarcoma-associated herpesvirus miRNA, miR-K12-11, downregulates a subset of cellular mRNAs also targeted by miR-155 which regulate cell cycle and apoptosis (17, 37).

Examination of well-characterized, virus-encoded miRNAs reveals that almost all exhibit specific temporal expression patterns and many are involved in the regulation of viral replication. Simian virus 40 miRNAs are encoded 3′ of the late pre-mRNA, accumulate late in infection and, by altering T-antigen presentation, regulate host immune response and increase the production of infectious virus (39). miRNAs derived from Marek's disease virus (8), Kaposi's sarcoma-associated herpesvirus (10), cytomegalovirus (CMV) (14, 18), and Epstein-Barr virus (EBV) (5, 32) all show temporal-specific expression, dominated by latency. Two recent studies have shown that endogenous expression of CMV-encoded miR-UL112-1 represses translation of the major CMV immediate early gene (IE1/IE72) and that premature expression significantly decreases viral DNA levels (18, 29). EBV miRNAs, perhaps the most well-studied suite of viral miRNAs, exhibit latent stage-specific infection, which may reflect their role in the regulation of viral replication (9, 32). Indeed, EBV-miR-BART2, which was originally predicted to target EBV DNA polymerase (32), has recently been shown to cleave viral DNA polymerase BALF5, reduce viral load, and inhibit transition from latent to lytic life cycles (5).

Here we report the characterization of the first miRNA derived from an insect ascovirus, the *Heliothis virescens* ascovirus (HvAV) (4), a double-stranded DNA virus and pathogen of prevalent lepidopteran pests. The genomes of HvAV and two other ascovirus species, *Spodoptera frugiperda* ascovirus (SfAV) (6) and *Trichoplusia ni* ascovirus (TnAV) (12), have been completely sequenced. Ascoviruses are enveloped virions (130 by 400 nm), allantoid to bacilliform in shape (7), whose pathology is characterized by host cell cleavage into virus-containing vesicles resembling apoptotic bodies (16). In insects, the vesicles are released into hemolymph, ultimately resulting in death of the host. The miRNA we have identified is derived from the major capsid protein (MCP) gene, and consistent with recent studies of viral miRNA function, targets HvAV DNA polymerase and regulates virus replication.

* Corresponding author. Mailing address: School of Integrative Biology, University of Queensland, St. Lucia, QLD 4072, Australia. Phone: (617) 3365 2043l. Fax: (617) 3365 1655. E-mail: s.asgari@uq.edu.au.

▽ Published ahead of print on 9 July 2008.

TABLE 1. Primers used for MCP and HvAV-miR-1 cloning and RNase III amplification[a]

Primer	Sequence
MCPmiR	5′-AAGTGGGCCTACCGA-3′
Linker miRNA	5′-TAGGCCAACCGCGAGAAGATG-3′
polyT+linker	5′-TAGGCCAACCGCGAGAAGATGTTTTTTTTTTTT-3′
MCP F	5′-CCGGATCCATGACTTCAAACGCCAGTGGTA-3′
MCP R	5′-GTCCGCGGTTAATTGAAATCGCCTCCGTT-3′
RNase III F	5′-GCGCGGATCCATGAGTGGGAATTCATTG-3′
RNase III R	5′-GCGCAAGCTTTTACACCTTTGAATTAGT-3′

[a] RNA nucleotides in the hybrid MCPmiR primer are underlined.

MATERIALS AND METHODS

Cells and virus infection. *Spodoptera frugiperda* cells (Sf9) were cultured in SF-900II serum-free media (Invitrogen) at 27°C as a monolayer. For virus infection, the medium was removed from the cells and replaced with ascovirus (HvAV-3e) inoculum for 1 to 2 h. Subsequently, fresh medium was added to the cells, which were maintained at 27°C until further analysis.

miRNA cloning. We used a previously described method for miRNA cloning, utilizing a DNA/RNA hybrid primer (26). Primers designed for miRNA cloning are given in Table 1. A 15-mer oligonucleotide was designed to the 5′ end of the predicted mature miRNA, HvAV-miR-1. RNA bases were incorporated at positions 4 and 5 of the oligonucleotide. The small RNA fraction was isolated from Sf9 cells at 48 hours postinfection (hpi) and 120 hpi, using a PureLink miRNA isolation kit (Invitrogen) according to the manufacturer's instructions. We used an NCode miRNA first-strand cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions to polyadenylate the small RNAs, followed by first-strand cDNA synthesis using a poly dT primer with a custom linker oligonucleotide. By using first-strand cDNA as a template, PCR was performed with MCPmiR and miRNA linker oligonucleotide primers (Table 1). The PCR conditions were 96°C for 3 min, 25 cycles of 96°C for 30 s, 41°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min. PCR products were run on a 4% agarose gel with a 10-bp DNA ladder (Invitrogen). A 65-bp fragment was eluted from the gel and cloned into the pGEM-T Easy vector system (Promega). Clones were sequenced and aligned with the MCP sequence.

Transient expression of MCP in insect cells. Full-length or truncated versions of HvAV MCP with (pIZ-MCP2; nt 898 to 1368 without an ATG start codon) or without (pIZ-MCP1; nt 1 to 897) the HvAV-miR-1 sequence were cloned into pIZ/V5-His (Invitrogen) under the control of a baculovirus immediate early gene promoter (see Fig. 2a). After confirming that MCP was oriented correctly in the vector, Sf9 cells were transfected with the plasmids (2 μg) using Cellfectin transfection reagent according to the manufacturer's instructions (Invitrogen). Expression of the miRNA was detected by Northern blot analysis (see below). With the pIZ-GFP vector, the efficiency of transfection averaged ~78%.

Northern hybridization and Southern blotting. Northern blot detection of HvAV-miR-1 was performed according to the Bartel lab protocol (http://web.wi.mit.edu/bartel/pub/protocols_reagents.htm). Briefly, 30 μg of total RNA per sample was separated on a 15% denaturing polyacrylamide gel and electroblotted to a nylon membrane using a semi-dry apparatus (Bio-Rad). Membranes were probed with a [γ-^{32}P]ATP end-labeled DNA oligonucleotide antisense to HvAV-miR-1. For a loading control, the same blots were stripped of previous probes by washing the membranes in 0.4 M NaOH at 42°C and rehybridized with a conserved *Drosophila* tRNA radiolabeled probe (GenBank accession no. V00229). Northern blot detection of MCP/DNA polymerase mRNA was accomplished by loading 10 μg of total RNA per sample on a 1% agarose gel with 2.2 M formaldehyde. RNA was transferred onto a nylon membrane using 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as previously described (35). Full-length PCR products of the gene of interest were end labeled with [α-^{32}P]dCTP and used as probes. Hybridization and membrane washes were carried out under high stringency conditions at 65°C. To remove probes, membranes were washed twice in boiled 0.1% sodium dodecyl sulfate and allowed to cool to room temperature.

For Southern analyses, total genomic DNA (10 μg) isolated from transfected Sf9 cells was transferred from a 1% agarose gel to a nylon membrane using 0.4 M NaOH. The membrane was probed overnight at 65°C with [α-^{32}P]dCTP-labeled, full-length HvAV RNase III, followed by high stringency washes at 65°C.

Expression of HvAV DNA polymerase in a baculovirus expression system. A set of primers was used to amplify a fragment 1,698 bp in length starting from nt 1561 to the end of the DNA polymerase gene, including the target sequence for HvAV-miR-1. The PCR product was then cloned in the Bac-to-Bac baculovirus expression vector pFastBac-HTb at the SacI and XbaI sites. All the procedures from transfection to fusion protein expression were conducted according to the manufacturer's instruction manual (Invitrogen). Expression of the recombinant protein was confirmed by analyzing protein expression by Western blotting, using an alkaline phosphatase-conjugated monoclonal antibody to His residues (1:5,000; Sigma).

Prediction of miRNAs and their targets. To predict potential stem-loop RNA structures transcribed from HvAV, ProMIRII (30) and Mfold (47) servers were used. To detect potential mRNA targets for HvAV-miR-1 within the HvAV genome, we used STarMir (http://sfold.wadsworth.org) (41) and miRBase (19) software. The parameters used were free energy of −25 kcal/mol and entropy of 1.8.

Identification of miRNA processing and regulatory proteins. Potential miRNA processing and regulatory proteins within the HvAV (4), *Bombyx mori* (28), and *S. frugiperda* (31) genomes were identified using an NCBI BLAST (45) similarity search with the *Drosophila* gene of interest. Homologues were subsequently interrogated with NCBI's CDART (conserved domain architecture retrieval tool) (45) to confirm conserved domain structures. To overcome the paucity of expressed sequence tag data available for *S. frugiperda*, we employed a sequential search strategy, first querying the *B. mori* database (SilkBase) with the *Drosophila* gene of interest, and then using the top *B. mori* hit to query the *S. frugiperda* database (SpodoBase). The *Drosophila* genes used in our query were as follows: Dcr-1 (GenBank accession no. NP_524453), Drosha (NP_477436), loquacious (NP_723813), pasha (NP_651879), and LIN-28 (NP_647983). We identified potential miRNA regulatory genes in the HvAV genome using CDART to identify protein domains conserved between HvAV genes and canonical *Drosophila* miRNA processing and regulatory proteins.

RESULTS

Prediction of a miRNA in the HvAV MCP. We queried the HvAV genome with ProMIRII and Mfold and identified several potential pre-miRNAs. We focused our analysis on a prediction in the sense strand of the MCP gene (Fig. 1b) with significant complementarity to HvAV DNA polymerase I (see below). The predicted precursor was found within the MCP open reading frame (ORF) from nt 911 to 1010, corresponding to genomic nt 59487 to 59508 (4).

Cloning and analysis of HvAV-miR-1. We used a recently described PCR method to clone the predicted mature miRNA from HvAV-infected Sf9 cells, a cell line derived from the lepidopteran *Spodoptera frugiperda* (26). Briefly, a 15-mer oligonucleotide complementary to the predicted mature miRNA (incorporating two RNA bases to increase specificity and relative melting temperature) was designed as a forward primer

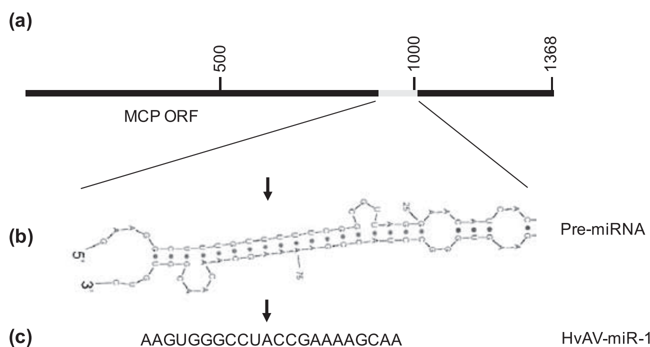

FIG. 1. Predicted miRNAs in the HvAV genome and stem-loop structure of pre-HvAV-miR-1. (a) Diagram showing the position of HvAV-miR-1 within the HvAV MCP ORF (shown in a gray box). (b) A potential pre-miRNA sequence forming a stem-loop structure in the sense strand of the MCP gene of HvAV from nt 911 to 1010 (genomic nt 59487 to 59508 [4]). (c) Mature HvAV-miR-1 sequence (nt 971 to 992).

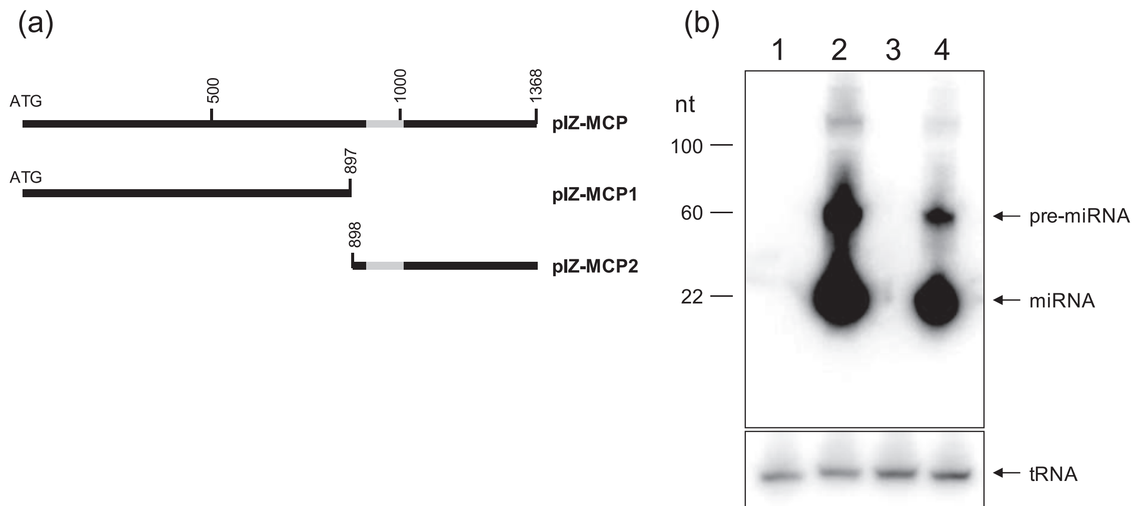

FIG. 2. Ectopic expression of HvAV-miR-1. (a) Schematic representation of MCP constructs with (gray line) and without HvAV-miR-1 sequence. Note that the MCP2 fragment cloned lacks the ATG start codon. (b) Sf9 cells were transfected with (1) pIZ/V5-His (empty vector), (2) pIZ-MCP, (3) pIZ-MCP1 without HvAV-miR-1 (nt 1 to 897), and (4) pIZ-MCP2 with HvAV-miR-1 (nt 898 to 1368). Cells were analyzed at 24 h posttransfection by Northern hybridization using a γ-^{32}P-labeled oligonucleotide (22-mer) reverse complementary to the HvAV-miR-1 sequence as a probe (above). Both pre-miRNA and mature HvAV-miR-1 were detected only in samples that contained the miRNA sequence. The membrane was rehybridized with a conserved *Drosophila* tRNA probe (below), after stripping the old probe, to demonstrate equal loading. Oligonucleotides of known nucleotide sizes were used as markers.

and paired with a reverse primer complementary to a custom linker. A specific 65-nt PCR product was amplified from the small RNA sample extracted from Sf9 cells at 120 hpi. The 22-nt miRNA sequence (AAGTGGGCCTACCGAAAAG CAA) was confirmed by sequencing and named HvAV-miR-1. We found homologous sequences of HvAV-miR-1 pre-miRNA in the MCP genes from the other two ascoviruses, SfAV and TnAV, with 68% and 50% similarity, respectively. However, applying the miRNA prediction software used to analyze the HvAV genome yielded no potential pre-miRNAs in MCP genes from the other two ascoviruses. However, this does not rule out the possibility of the presence of potential miRNAs from TnAV and SfAV outside their MCP genes.

To investigate if HvAV-miR-1 is expressed independent of viral infection, the full-length gene coding for HvAV MCP was transiently expressed in Sf9 cells under the control of a baculovirus early promoter (pIZ-MCP). Northern blot analysis showed expression of the miRNA at 24 h posttransfection (Fig. 2b, lane 2). However, when cells were transfected with the empty vector or the modified ORF lacking the miRNA sequence (Fig. 2a; pIZ-MCP1, from nt 1 to 897), HvAV-miR-1 was not detected (Fig. 2b, lanes 1 and 3, respectively). The miRNA was detected when a truncated MCP ORF containing the miRNA (Fig. 2a; pIZ-MCP2, from nt 898 to 1368) was cloned in the vector and transfected into Sf9 cells (Fig. 2b, lane 4).

HvAV-miR-1 expression. We investigated the temporal expression of HvAV-miR-1 and MCP by Northern blotting using total RNA isolated at 0 h, 16 h, 24 h, 48 h, 72 h, 96 h, 120 h, and 144 h following Sf9 cell HvAV infection. Small RNAs corresponding to both the pre-miRNA (~60 nt) and mature HvAV-miR-1 (22 nt) were observed at 96 to 144 hpi (Fig. 3a). In contrast, MCP expression was detected as early as 24 hpi (Fig. 3b) and as late as 144 hpi (3), suggesting that MCP transcripts

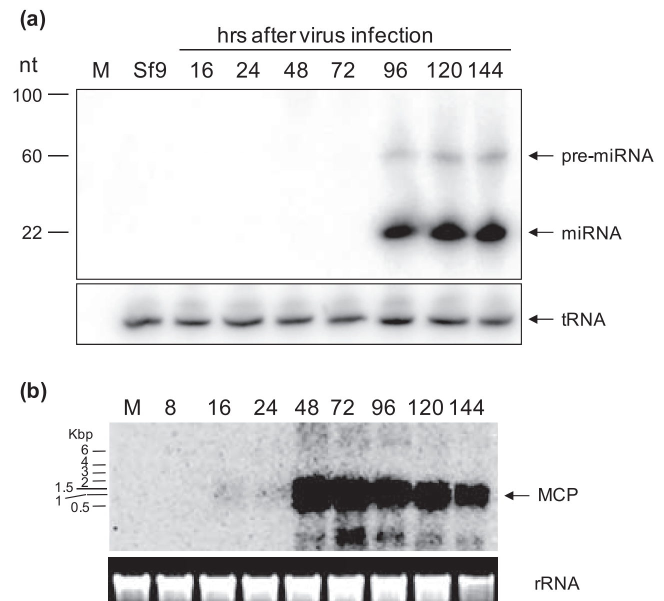

FIG. 3. Time course expression analyses of HvAV-miR-1 and MCP transcripts in Sf9 cells infected with HvAV. (a) miRNA Northern analysis for the detection of HvAV-miR-1 in virus-infected Sf9 cells. The γ-^{32}P-labeled oligonucleotide (22-mer) reverse complementary to the HvAV-miR-1 sequence was used as a probe. MCP pre-miRNA (~60 nt) and HvAV-miR-1 (22 nt) were detected from 96 hpi. Oligonucleotides of known nucleotide sizes were used as markers. The membrane was rehybridized with a conserved *Drosophila* tRNA probe (below) after removing the old probe to demonstrate equal loading of samples. (b) Northern analysis using α-^{32}P-labeled full-length MCP as a probe to detect MCP mRNA. Expression starts from 16 hpi with the highest level at 48 hpi and continues to late hpi. Total RNA from mock-inoculated (M) Sf9 cells was used as a negative control. The gel was stained with ethidium bromide before transfer to confirm equal loading of all samples (shown by 18S rRNA).

may be extremely stable or are equivalently expressed at many time points. To investigate if host genes could regulate HvAV-miR-1 expression, we queried the *S. frugiperda* (host) and *Bombyx mori* (a distantly related lepidopteran species) genomes for homologues of *Drosophila* miRNA processing genes. We identified *B. mori* clones representative of loquacious/TRBP (SilkBase entry fner29p11f) and Dicer-1 (SilkBase entry FWDP01_FL5_J09) annotated with the gene ontology terms "pre-miRNA processing" and "RNase III activity," respectively. Although the *S. frugiperda* database contains relatively few expressed sequence tags, we identified *B. mori* and *S. frugiperda* homologues of pasha/DGCR8 (SilkBase entry wdS00697; SpodoBase entry SF9L08090) and LIN-28 (SilkBase entry fe100P15_F_D15; SpodoBase entry Sf1F07186-3-1). Surprisingly, a definitive clone for Drosha could not be identified in either organism, although we suspect this reflects the shallow depth of the databases and not the true absence of the gene. We also queried the HvAV genome and, consistent with the original genome annotation (4), identified a gene with an RNase III domain (*orf27*) and a second with a DEAD-like helicase domain (*orf15*), both of which are homologous to domains in Dicer-1 and Drosha. These results indicate that the protein machinery necessary for tight regulation of HvAV expression is present in Sf9 cells and that either host- or virus-encoded genes could participate in HvAV-miR-1 processing. We reasoned that HvAV-miR-1, particularly because it is only present at late time points after HvAV infection, may play an important role in the viral life cycle.

Role of HvAV-miR-1 in virus DNA replication. We used STarMir to identify potential HvAV-miR-1 targets and found a significant antisense match between the majority of the HvAV-miR-1 seed region (nt 3 to 8) and HvAV DNA polymerase I (*orf1*) (Fig. 4a). miRNA seed sequences, nt 2 to 8, are important for mRNA target recognition (24); however, research has shown that perfect seed pairing is not a robust predictor of in vivo interaction (13).

We investigated the temporal expression of *orf1* in host cells following HvAV infection and found a sharp decline in *orf1* after 96 hpi (Fig. 4b), the same time point at which HvAV-miR-1 is first detected. To investigate if HvAV-miR-1 is able to affect virus replication by targeted downregulation of *orf1*, MCP was ectopically expressed in Sf9 cells prior to HvAV inoculation. We assessed the effect of constructs containing the complete MCP ORF (pIZ-MCP), two truncated versions without (pIZ-MCP1) and with (pIZ-MCP2) the HvAV-miR-1 sequence, and the empty vector (pIZ). We confirmed expression of HvAV-miR-1 in cells transfected with constructs containing the miRNA sequence (pIZ-MCP and pIZ-MCP2) (Fig. 2). Forty-eight hours following inoculation of transfected cells, total viral load was assessed by Southern blotting, using a probe against HvAV RNase III (*orf27*). We found a significant reduction of viral DNA when cells were transfected with constructs containing the miRNA sequence (pIZ-MCP and pIZ-MCP2) 24 h prior to infection compared to the level in cells that were transfected with pIZ empty vector or the construct without HvAV-miR-1 sequence (pIZ-MCP1) (Fig. 5a).

To test if HvAV DNA polymerase transcripts are specifically targeted by HvAV-miR-1, we performed a series of independent experiments assessing the abundance of *orf1* transcript levels in Sf9 cells in the presence of pIZ, pIZ-MCP, pIZ-MCP1, and pIZ-MCP2 expression constructs. Twenty-four hours after transfection, cells were infected with HvAV. We assessed the abundance of *orf1* transcript levels 72 h after infection by Northern hybridization and found no *orf1* expression in cells transfected with MCP constructs that contained the miRNA sequence (pIZ-MCP and -MCP2; Fig. 5b). Considering that the MCP2 fragment lacked an ATG codon, it is clear that downregulation of *orf1* transcript levels is not due to MCP protein but due to expression of HvAV-miR-1. Furthermore, when the same blot was hybridized with another probe specific to the HvAV *bro11* gene (*orf98*) after removal of the initial probe, a *bro11*-specific band was detected in all samples (Fig. 5b).

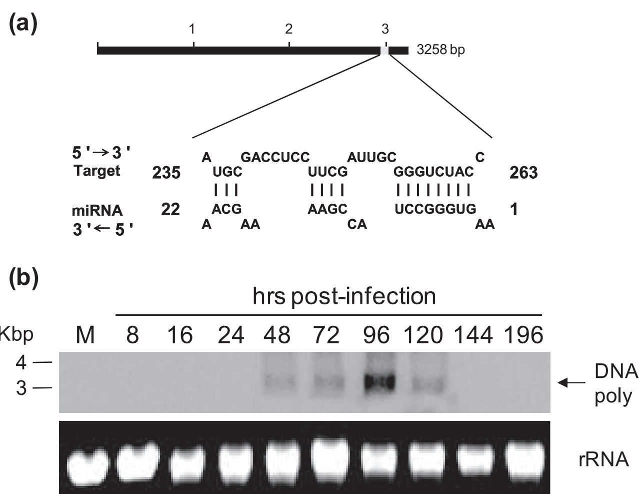

FIG. 4. HvAV-miR-1 targets HvAV DNA polymerase I. (a) Schematic diagram showing the position of the HvAV-miR-1 target sequence in the HvAV DNA polymerase gene (nt 2998 to 3026). The seed region of HvAV-miR-1 matches the HvAV DNA polymerase sequence at the 5′ end. (b) Northern analysis of total RNA extracted from Sf9 cells at various times following infection. The α-^{32}P-labeled full-length ORF coding for HvAV DNA polymerase was used as a probe. The blot shows detection of a 3.2-kb mRNA specific to the HvAV DNA polymerase gene. Expression starts at 24 hpi and diminishes from 96 hpi.

In addition, we produced a recombinant baculovirus expressing a fragment of the HvAV DNA polymerase I gene containing the target sequence for HvAV-miR-1. The protein was produced in fusion with six-histidine residues as a tag. Sf9 cells previously transfected with pIZ empty vector, pIZ-MCP, pIZ-MCP1 (lacking HvAV-miR-1), and pIZ-MCP2 (containing HvAV-miR-1) were infected with the recombinant baculovirus. At 72 hpi, cells were collected and analyzed by Western blotting in which an anti-His monoclonal antibody was used as a probe. Expression of a 69-kDa fusion protein corresponding to the HvAV DNA polymerase fragment was significantly reduced in cells previously transfected with vectors containing HvAV-miR-1 sequence (Fig. 6a; pIZ-MCP and pIZ-MCP2) relative to the ones transfected with vectors lacking the miRNA (Fig. 6; pIZ and pIZ-MCP1). The presence of a very weak signal in pIZ-MCP and pIZ-MCP2 treatments was presumably from a small proportion of cells that may not have received the plasmids, as the efficiency of transfection was determined as averaging ~75%, using green fluorescent pro-

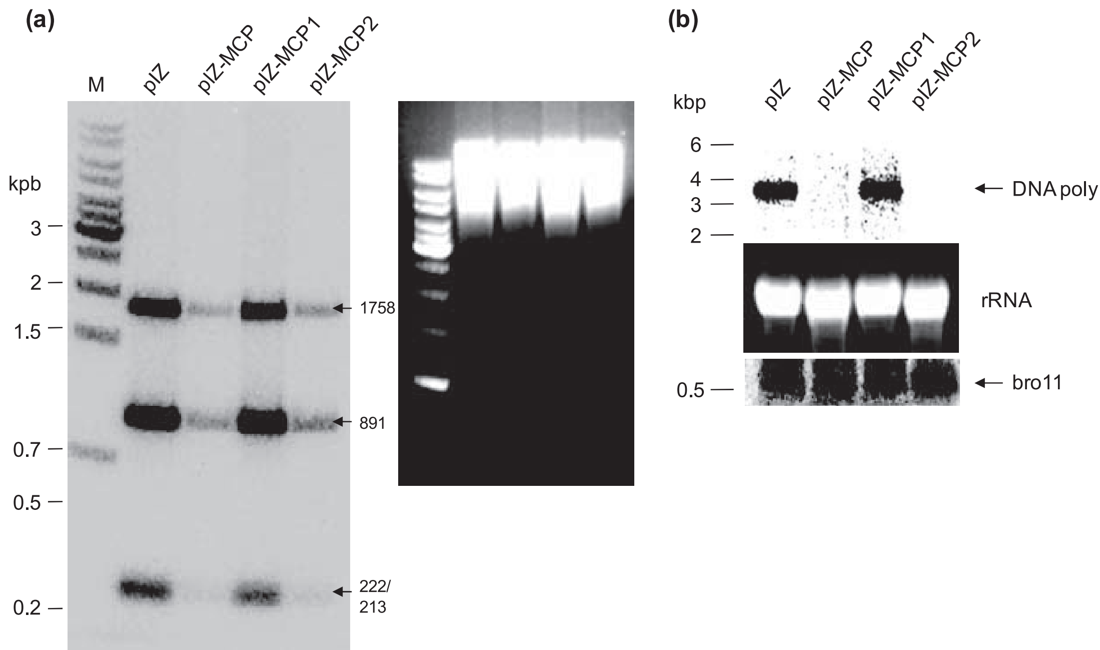

FIG. 5. HvAV-miR-1 affects virus replication by targeting HvAV DNA polymerase I. (a) Southern blot analysis of total genomic DNA (10 μg) from Sf9 cells 48 h after HvAV infection which were transfected (24 h prior to inoculation) with pIZ empty vector, pIZ-MCP, pIZ-MCP1 (lacking HvAV-miR-1), and pIZ-MCP2 (containing HvAV-miR-1). Genomic DNA was digested with EcoRI prior to analysis. A DNA fragment from the HvAV RNase III gene was used as the probe. Three expected HvAV RNase III EcoRI fragments (bp 1758, 891, and 222/213) were detected in the blot. Signal intensity from the band corresponding to HvAV RNase III was strongly reduced in infected cells that were previously transfected with pIZ-MCP or pIZ-MCP2 compared to the level in cells transfected with the empty vector (pIZ) or pIZ-MCP1, indicating a significant reduction in virus replication. M, molecular marker. The corresponding agarose gel is shown on the right. (b) Northern blot analysis of total RNA from Sf9 cells treated as in panel "a" in an independent experiment. The blot was initially probed with an *orf1*-specific probe (upper blot). No *orf1* corresponding transcript (3,261 bp) was detected in infected cells that were previously transfected with pIZ-MCP or pIZ-MCP2, whereas transcripts were detected in cells transfected with the empty vector (pIZ) or pIZ-MCP1, indicating that the miRNA specifically targets *orf1* transcripts. After stripping the probe, the same blot was hybridized with another probe specific to the HvAV *bro11* gene (lower blot). Signal was detected in all samples, indicating that *orf1* is specifically targeted by HvAV-miR-1.

tein expression as a marker. The same Western blot was probed with a monoclonal antibody specific to the glycoprotein 64 (gp64) from *Autographa californica* multiple nucleopolyhedrovirus to ensure that HvAV-miR-1 did not affect replication of the recombinant baculovirus (Fig. 6b). The signal strength corresponding to gp64 was equal in all samples. These results, and the others presented above, strongly support the hypothesis that HvAV-miR-1 can inhibit virus replication by specifically downregulating *orf1*.

DISCUSSION

The search for virus-encoded miRNAs and the study of their function has led to profound insights into virology (reviewed in references 15, 36, and 38). In this study, we searched for putative miRNA stem-loop precursors encoded by an insect ascovirus, HvAV, and focused our research on the isolation and characterization of a single prediction (HvAV-miR-1), encoded within a protein-coding region of the MCP.

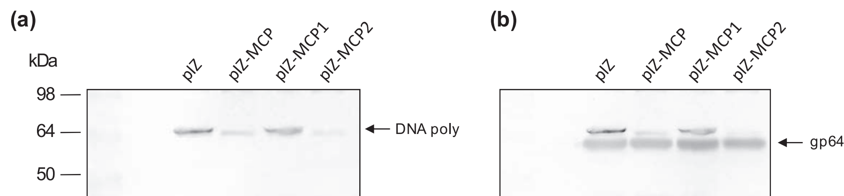

FIG. 6. HvAV-miR-1 specifically targets HvAV DNA polymerase I. (a) A recombinant baculovirus was constructed expressing a fragment of the HvAV DNA polymerase I gene containing the target sequence for HvAV-miR-1. Sf9 cells were initially transfected with pIZ empty vector, pIZ-MCP, pIZ-MCP1 (lacking HvAV-miR-1), and pIZ-MCP2 (containing HvAV-miR-1). Twenty-four hours following transfection, cells were infected with the recombinant baculovirus. At 72 hpi, cells were collected and analyzed by Western blotting in which an anti-His monoclonal antibody was used as a probe (1:5,000). Expression of a 69-kDa protein corresponding to the HvAV DNA polymerase fusion protein was significantly reduced in cells transfected with vectors containing the HvAV-miR-1 miRNA (pIZ-MCP and pIZ-MCP2). (b) The blot in panel "a" was reprobed with a monoclonal antibody to the *Autographa californica* multiple nucleopolyhedrovirus gp64 protein (1:5,000) to show that the miRNA did not affect replication of the virus but specifically affected expression of the recombinant HvAV DNA polymerase.

After successfully detecting the predicted mature HvAV-miR-1, we investigated its temporal expression. By using a recently described miRNA cloning strategy and Northern blot analyses, we found that HvAV-miR-1 is specifically expressed late in virus infection, despite nearly constitutive expression of MCP. Consistent with these findings, several recent studies have also reported stage-specific expression of viral miRNAs (5, 8, 11, 14, 18, 32, 39). However, the regulatory mechanism(s) underlying the HvAV-miR-1 expression and other virally encoded miRNAs has yet to be elucidated. Our results show that the proteins necessary for the temporal regulation of HvAV-miR-1 are present in *S. frugiperda* cells and that two proteins encoded in the HvAV genome could participate in HvAV-miR-1 processing. Recent studies have reported that pasha/DGCR8 and loquacious/TRBP are necessary for correct mature miRNA processing (for reviews, see references 22, 23, and 42), potentially implicating them in HvAV expression.

Regulated processing of primary miRNA precursors has been observed in undifferentiated tissues, including embryonic stem cells, embryonal carcinomas, and primary tumors (40). For example, a comprehensive analysis of miRNA expression in embryonic stem cells revealed that pri-miRNA processing was inhibited at the Drosha step (40). Recently, Lin28 has been identified as the primary negative regulator of Drosha processing for a large subset of mouse pri-miRNAs (44). Interestingly, this regulation is temporally dependent and restricted to undifferentiated embryonic stem cells or cancer cells, which are "de-differentiated" (44). We suggest a model based on these studies in which HvAV replication mimics cellular differentiation, and the *S. frugiperda* LIN-28 homologue is co-opted soon after infection to inhibit HvAV-miR-1 processing. This preliminary model would successfully explain HvAV-miR-1's temporal expression and would suggest that the high levels of HvAV-miR-1 observed shortly after transfection could be due to a limiting amount of Lin28. We cannot rule out, however, that the HvAV-encoded RNase III and DEAD helicases may also play a role in regulating HvAV-miR-1, either by competition or cooperation with the host miRNA processing machinery.

We identified possible targets for HvAV-miR-1 computationally and found a significant antisense match to HvAV DNA polymerase I. Indeed, DNA polymerase I transcript levels are significantly decreased when endogenous HvAV-miR-1 is expressed, consistent with reports of miRNA-directed endonucleotic cleavage (1, 21, 25, 43, 46), including EBV-miR-BART-2 (see below) (5, 32). Although cleavage is usually favored by perfect base-pairing between the miRNA and the mRNA, mismatches can be tolerated (20, 27, 46). To investigate if HvAV-miR-1 directly downregulates DNA polymerase I, and therefore inhibits viral replication, we performed a series of experiments ectopically expressing three MCP variants. Only constructs containing HvAV-miR-1 inhibited DNA polymerase I expression and virus replication. Our results are in striking congruence with reports of EBV-encoded miRNA (miR-BART2) regulation of EBV DNA polymerase by degradation (5, 32) and suggest that virus-encoded miRNAs may function generally to regulate virus life cycles.

We propose that viruses have evolved mechanisms to utilize host RNA interference machinery for their own regulation. Indeed, miRNAs may allow fine-scale control of temporal expression patterns, thus tightly regulating virus replication, preventing the rapid decline or death of the host, and therefore conferring a selective advantage resulting in greater numbers of viable virus progeny. To our knowledge, HvAV-miR-1 is the first miRNA characterized from an insect virus. Further functional analysis of HvAV-miR-1, and other potential miRNAs encoded by the HvAV genome, will be facilitated by the development of tools to genetically manipulate ascoviruses. Undoubtedly, these efforts will clarify the role of these small molecules in virus regulation and may lead to novel RNA-targeted pesticides.

ACKNOWLEDGMENTS

This project was supported by a University of Queensland internal grant to S. Asgari and a UQ Ph.D. Scholarship to M. Hussain. R. J. Taft is supported by a United States National Science Foundation Graduate Research Fellowship.

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