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The Early Gene *hhi1* Reactivates *Heliothis zea* Nudivirus 1 in Latently Infected Cells[▽]

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***Heliothis zea* nudivirus 1 (*Hz*NV-1), previously known as Hz-1 virus, is an insect virus able to establish both productive and latent infections in several lepidopteran insect cells. Here, we have cloned and characterized one of the *Hz*NV-1 early genes, *hhi1*, which maps to the HindIII-I fragment of the viral genome. During the productive viral infection, a 6.2-kb *hhi1* transcript was detectable as early as 0.5 h postinfection (hpi). The level of transcript reached a maximum at 2 hpi and gradually decreased after 4 hpi. The transcript was not detectable during the latent phase of viral infection. Upon cycloheximide treatment, much higher levels of *hhi1* transcript were detected throughout the productive viral infection cycle, suggesting that newly synthesized proteins are not needed for the expression of *hhi1*. Nevertheless, viral coinfection can further stimulate the expression of transfected *hhi1* promoter in a plasmid. Transient *hhi1* expression in latently infected cells resulted in a significant increase in virus titer and viral DNA propagation, suggesting that *hhi1* plays a critical role in viral reactivation. Additional experiments showed that six early genes, which possibly function in transcription or DNA replication, were activated in the latent cells upon *hhi1* transfection. Among these six genes, *orf90* and *orf121* expression could be induced by *hhi1* alone without the need for other viral genes. Our discovery should be useful for future mechanistic study of the switches of latent/productive *Hz*NV-1 viral infections.**

Virus infection of host cells usually results in productive infection in which the viruses express most, if not all, of their genes, replicate their genomes, and finally release progeny viruses. In most cases, host cells are killed by the productive viral infection (3, 14). In latent infections, such as those caused by herpes viruses in nerve tissues, the virus is quiescent, and no infectious virus particles can be detected. Periodic reactivation results in the production of viral progeny and transmission to new hosts (6).

Latent viral infections are difficult to observe and study; consequently, these phenomena are usually neglected and rarely reported (3, 18, 30). In addition, due to the difficulty in establishing latent viral infections in laboratory stocks of culture cells or insects, latent viral infections are usually identified only after an unexpected viral reactivation from previously healthy-looking insects or insect cells (24). So far, we still know very little about genes and mechanisms governing the switch of latent/productive viral infections in mammals and insects.

Heliothis zea nudivirus (*Hz*NV-1), originally named Hz-1 virus, was first found to be a latent virus in the IMC-Hz-l cell line isolated from ovarian tissue of *H. zea* (8, 10, 14, 16, 21). This virus has been reported to infect many insect cell lines including *Trichoplusia ni* (TN-368), *Spodoptera frugiperda* (IPLB-SF-21), *H. zea* (IPLB-1075), *Mamestra brassicae*, and *Porthetria dispar* (IPLB-65Z) (1, 8, 22, 25) and to establish latent infections in most of these insect cell lines, which are permissive to this virus. *Hz*NV-1 virus is an enveloped, rod-shaped, nonoccluded virus with a circular double-stranded DNA genome of 228 kb, which is encased in a 414 ± 30 nm nucleocapsid (1, 3, 4, 11). The *Hz*NV-1 genome has been completely sequenced and is estimated to contain 154 open reading frames (ORFs) (5). Previously, it was classified as a member of the baculovirus family, but due to its lack of an occlusion body and low sequence homology to a typical baculovirus, it has now been relegated to a new nonoccluded genus, the *Nudivirus* (27, 28).

*Hz*NV-1 virus has two infection cycles, latent and productive, in its life cycle. It can infect insect cells and stay latent for many passages (3, 19, 30). Studies of TN-368 and Sf-21 cells have shown that during the productive infection stage, most cells are

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killed, and high titers of virus progeny are produced; however, often a small percentage of the cells, usually less than 5%, become latently infected, and virus persists for a prolonged period of time within these cells (3, 30). During latency, virions were not detectable in most of these latently infected cells, and viruses existed either as episomes or inserted into the host genome (19). Sometimes virus particles can be released from very small proportions (usually less than 0.2%) of latently infected cells, resulting in the death of these cells. This small quantity of continuously released virions results in the presence of low viral titers (around 10^3 PFU/ml) in the culture medium of latently infected cells (4, 19).

The temporal gene expression profiles of the *Hz*NV-1 virus during latent and productive infection have been analyzed. During latent infection, persistency-associated transcript 1 (PAT1), which is expressed by persistency-associated gene 1 (*pag1*), was found to be involved in the establishment of latent infection of *Hz*NV-1 virus and was the only detectable transcript during latent infection (3, 4). During productive viral infection, an abundant 6.2-kb transcript was detected from the HindIII-I fragment of the genome of *Hz*NV-1. The gene was identified and named *hhi1* as it is the first gene identified in the *Hz*NV-1 virus *H*indIII-*I* fragment (3, 4, 31). Wu et al. (31) found that *hhi1* promoter can be activated in Sf-21 cells by the baculovirus, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV). In this study, we further characterized the temporal expression of *hhi1* and its function. We found that de novo protein synthesis is not required for *hhi1* gene expression upon viral infection. The *hhi1* gene is shown to be capable of reactivating viruses from latently infected cells. This discovery highlights *hhi1* as a key gene useful for further mechanistic studies of viral gene regulation and switching during latent and productive viral infections.

MATERIALS AND METHODS

Cells and virus. *Spodopter frugiperda* IPLB-Sf-21 was incubated in TC-100 insect cell culture medium, which contained 10% fetal bovine serum (FBS) at 26°C (Gibco BRL, Gaithersburg, MD) (23, 31). Standard *Hz*NV-l virus was derived by a serial dilution of the stock viral solution and isolated by plaque purification. SFP4 cells were derived from latently infected Sf-21 cells (3, 4). The titers of the virus clones were estimated by both quantitative PCR (qPCR) (20) and 50% tissue culture infective dose ($TCID_{50}$) (23).

Computer-assisted sequence analysis. The contiguous 10,878-bp HindIII-I fragment was sequenced and assembled using Phrep/Phrap and Sequencer software, version 4.1.1 (Sequencher, Ann Arbor, MI). The ORF prediction was performed using GenScan (http://genome.dkfz-heiderlberg/de/cgi-bin/GENSCAN/) and the National Center for Biotechnology Information (NCBI) ORF Finder program (http://www.ncbi.nlm.nih.gov/). The nucleotide and amino acid sequences were analyzed using the NCBI BLAST program and further confirmed by the sequencing results of Cheng et al. (5).

RNA isolation and Northern blot analysis. Sf-21 cells were inoculated at a multiplicity of infection (MOI) of 10 with *Hz*NV-l virus and incubated at 26°C for 2 h with gentle rocking. After adsorption, total cellular RNA samples were extracted from productively infected cells at 0.5, 1, 2, 4, 6, 8, 10, and 12 h postinfection (hpi) and from latently infected SFP4 cells using Ultraspect RNA (Bioteck, Houston, TX). In some experiments, cycloheximide (50 μg/ml; Sigma, St. Louis, MO) or actinomycin D (10 μg/ml; Sigma) was added to the cells 1 h prior to viral infection or at the indicated times (see Fig. 2) and maintained at the same concentration during the course of experiments. Briefly, approximately 15 μg of total RNA (Qiagen) extracted from virus-infected SF-21 or SFP4 was loaded, blotted onto Hybind-N+ nylon transfer membrane (GE Healthcare), and hybridized with *hhi1*- or *pag1*-specific probe, which was labeled with digoxigenin (DIG) by PCR amplification of template *Hz*NV-1 viral DNA and using a PCR DIG Probe Synthesis Kit (Roche Applied Sciences, Burgess Hill, United Kingdom) according to the manufacturer's instructions.

Plasmid construction. The 10.8-kb HindIII-I fragment of *Hz*NV-l viral genomic DNA was cloned into the HindIII site of the low-copy-number vector pWSK29 (26). A DNA fragment of 4,518 nucleotides from bp 222659 to 227176 of *Hz*NV-1 virus was PCR amplified using the following primers: HHI1 F, 5′-ATTCCCGGGCTCTCCTCTACAATCATGTCTACCGTG-3′; HHI1 R, 5′-ATTCCCGGGCTCAGATTCACAGTATGGTTCACG-3′ (restriction sites are underlined). The amplified fragment was cloned into T-vector and subsequently released by SmaI cleavage and inserted downstream from the *Drosophila* heat shock promoter (15) in plasmid pBluescript (Stratagene, La Jolla, CA). The resulting plasmid was pKShH1. We obtained *egfp*, *orf60*, *orf75*, *orf90*, *orf101*, *orf121*, and *orf131* coding regions by PCR with SmaI and inserted them downstream of the *hsp70* promoter (*p-hsp*) of plasmid pKShsp70 to give the expression plasmids.

Different *Hz*NV-1 viral early promoter regions, *hhi1* (+35 to −688) (31), *pag1* (+29 to −727), and *orf60*, *orf75*, *orf90*, *orf101*, *orf121*, and *orf131* promoters (+1 to −500) were obtained by PCR using *Hz*NV-1 viral DNA as a template. Promoter regions were ligated into pGL3-basic vector (Promega, Madison, WI) to obtain plasmids pGL3-*hhi1* and pGL3-*pag1* for activity assay with or without *Hz*NV-1 infections and to obtain plasmids pGL3-*orf60*, pGL3-*orf75*, pGL3-*orf90*, pGL3-*orf101*, pGL3-*orf121*, and pGL3-*orf131*, respectively, for activity assays upon *hhi1* cotransfection.

DNA transfection into cells. A total of 2×10^5 cells were seeded in a 24-well culture plate (Corning, Acton, MA) and then transfected with 0.5 μg of appropriate plasmid DNAs using Cellfectin (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol (Gibco BRL). The promoter activities, cellular morphology, or the virus titers of the transfected cells were studied at the indicated time points (see Fig. 4) in different experiments.

Luciferase activity assay. Luciferase assays were conducted as described previously (31, 32). Luciferase activity was measured with a luminometer (Lumat LB 9501; Berthold) by injecting 50 μl of 0.2 mM luciferin (Promega) into each well. The results were plotted as average luciferase activity versus time of infection from triplicate assays of three independent experiments.

Quantitation of viral early gene expression. Virus RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany). The RNA pellet was dissolved in 30 μl of diethyl pyrocarbonate (DEPC) and used for cDNA synthesis. cDNA synthesis was performed using SuperScript III first-strand synthesis system for reverse transcription-PCR (RT-PCR), following the protocol from the manufacturer (Invitrogen). *Hz*NV-1 early genes were RT-PCR amplified using the following primers: p60 R, 5′-ATTAGATCTATGGCTGTATACCACTAGTGT-3′; p60 F, 5′-ATTGGTACCACTGGGCATCACGTTGTTAC-3′; p75 R, 5′-ATTAGATCTATTTAGAAAGATGATT-3′; p75 F, 5′-ATTGGTACCACCGGTCAAGAGTACATTGG-3′; p90 R, 5′-ATTAGATCTATGTTTGCTGCTGAACGCTA-3′; p90 F, 5′-ATTGGTACCTGAGTGATTTGTCATACTTG-3′; p101 R, 5′-ATTAGATCTCTTATCAGCAAATGTTGAT-3′; p101 F, 5′-ATTGGTACCTTGTACCACTGTCGAGTCCA-3′; p121 R, 5′-ATTAGATCTATGTTAAGAGTCTAAAA-3′; p121 F, 5′-ATTGGTACCGAACTTTAAGACTTGAGAAT-3′; p131 R, 5′-ATTAGATCTTCGTGACTACTAAACCACAA-3′; and p131 F, 5′-ATTGGTACCCCTCAAAACACGTATAAATC-3′.

Briefly, amplification was carried out by adding 1 μl of cDNA in the Master Mix *Taq* polymerase (MBI Fermentas, Vilnius, Lithuania). The resulting DNA products (Amplicon, Brighton, United Kingdom) were analyzed on agarose gel (1.5%) after electrophoresis at 100 V for 30 min.

RNA interference. For *hhi1* knockdown experiments, all small interfering RNAs (siRNAs) were predicted and synthesized by MDbio Inc. (MDbio, Taiwan). The siRNAs used in this study were *hhi1* siRNA (5′-CGUUCUGAAAACGACGUAAGAUUAGA-3′) and *egfp* siRNA (5′-GGCGAUGCCACCUACGGCAAG-3′). Sf-21 cells (4×10^4) in 96-well plates were transfected with 50 nM siRNA using a Silencer siRNA Transfection Kit II (Applied Biosystems). Cells were transfected with designed siRNAs, and then at 4 h posttransfection (hpt), the cells were infected with *Hz*NV-1 virus (MOI of 1), and the number of latently infected colonies was again calculated at 12 days postinfection (4). Several primers were designed for the RT-PCR amplification of *hhi1*, *pag1*, and *actin* as follows: hhi1-1F, 5′-CGATATGAACATTAACGATGACGATC-3′; hhi1-1R, 5′-AAACGGATGCAAAATGGACTCAA-3′; pag1-F, 5′-ACGGGAATTCAGTGTCGAGGACTT-3′; pag1-R, 5′-CATGTCTAGAACCCTACCTACCT-3′; actin-F, 5′-CGTGATGGTGGGCATGGGTCAG-3′; and actin-R, 5′-CTAATGTCACGCACGTATTCC-3′.

Nucleotide sequence accession number. The nucleotide sequence of the *hhi1* gene was submitted to the GenBank database under accession number AF264019.

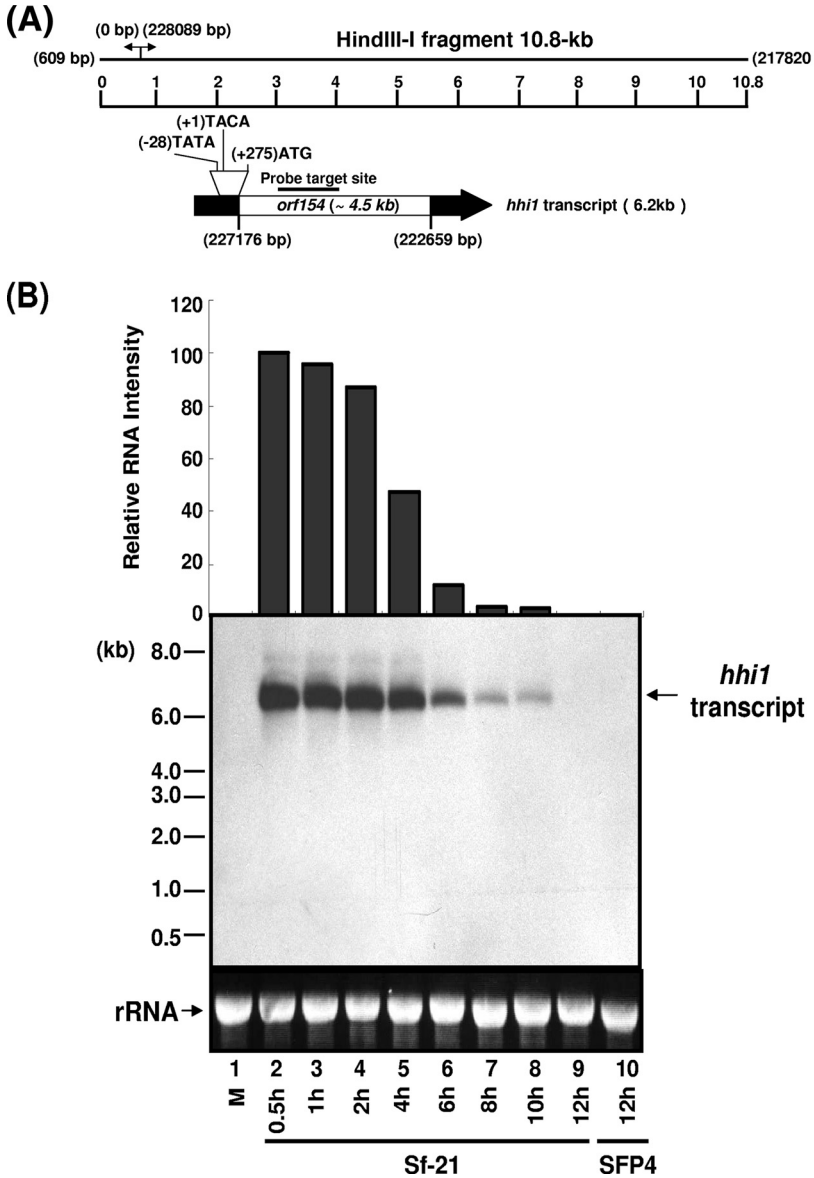

FIG. 1. Mapping of the *hhi1* transcript of *Hz*NV-1 virus. (A) Mapping of *hhi1* transcript. The first line shows the corresponding sequence numbers, 609 bp and 217820 bp on the complementary strand, of the nucleotides of HindIII-I fragment (5). The locations of a TATA box-like sequence, initiation site of transcription (+1), and translational start codon (ATG) on the HindIII-I fragment are indicated. (B) Temporal expression profile of the *hhi1* gene. Sf-21 cells were infected with *Hz*NV-1 virus at an MOI of 10. Total RNA was extracted and resolved on agarose gel and analyzed by Northern blot analysis using an *hhi1*-specific probe generated from a DIG labeling reaction. Lane 1, total RNA extracted from mock-infected cells; lanes 2 to 9, total RNA extracted from infected Sf-21 cells at 0.5 to 12 hpi; lane 10, total RNA extracted from latently infected SFP4 cells.

RESULTS

The *hhi1* gene maps to a 10.8-kb HindIII-I fragment of the *Hz*NV-1 viral genome. Previously, we reported a 6.2-kb early transcript mapped to the HindIII-I region (10.8 kb) of the *Hz*NV-1 viral genome (3); the gene encoding this 6.2-kb transcript was named *hhi1* (Fig. 1A). The temporal expression pattern and the orientation of *hhi1* transcript were studied. Sf-21 cells were infected with *Hz*NV-1 virus at an MOI of 10. Total cellular RNA was extracted from these productively infected cells at the indicated time points (Fig. 1B). Total cellular RNA was also isolated from uninfected Sf-21 cells to serve as a negative control. Northern blot analysis showed that a transcript was detected very early at 0.5 hpi. The levels of the transcript remained high from 0.5 hpi to 4 hpi and then decreased gradually. The gene was almost undetectable after 10 hpi (Fig. 1B). Due to its early appearance, it is regarded as an early transcript. This result was further confirmed by RT-qPCR with primers specific for the *hhi1* gene, which showed a similar pattern of transcript expression and disappearance (data not shown).

Analysis of *hhi1* expression in the presence of cycloheximide. To study whether *hhi1* expression requires viral protein synthesis, cycloheximide, an antibiotic which blocks translation, was added to the cell culture medium. After viral infec-

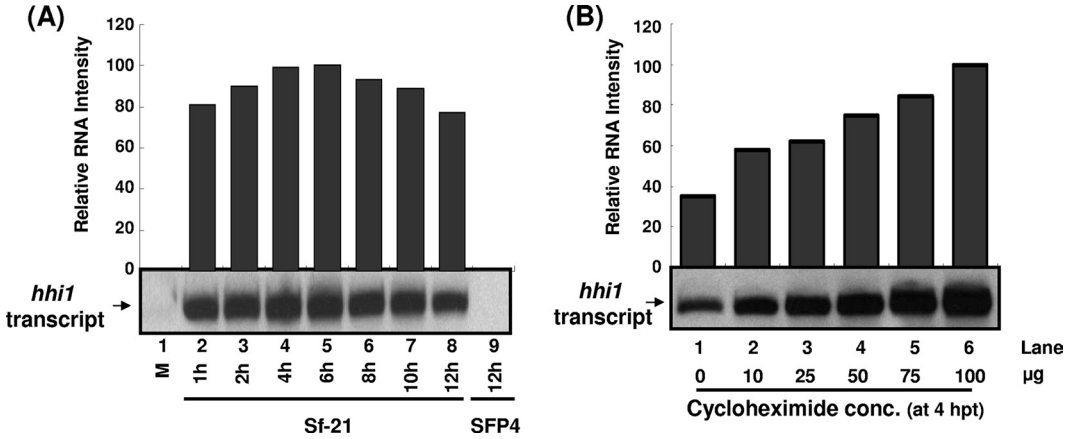

FIG. 2. Effect of cycloheximide on *hhi1* expression in *Hz*NV-1-infected cells. (A) Temporal expression pattern of *hhi1* transcripts in the presence of cycloheximide. Cells were treated with cycloheximide (50 μg/ml) 1 h prior to infection with *Hz*NV-1 virus, and total RNA was extracted at different time points postinfection. These RNA samples were analyzed by Northern blotting using DIG-labeled probe specifically for *hhi1* transcript. (B) *hhi1* transcript accumulation observed by the treatment of cycloheximide at different concentrations.

tion, total cellular RNA was extracted from cells and analyzed by Northern hybridizations. The *hhi1* transcript was detected throughout the infection cycle in the presence of cycloheximide (50 μg/ml), with a maximal accumulation of *hhi1* transcript at 4 to 6 hpi, which then fell slightly at later stages of infection (Fig. 2A). Concentration-dependent experiments showed that HHI1 expression increased proportionally to the increasing concentrations of cycloheximide, suggesting that protein synthesis had a negative effect on *hhi1* gene expression (Fig. 2B). These data indicate either that *hhi1* expression is blocked or that its transcript is quickly degraded at later stages of viral infection. We also noted that there is no sign of *hhi1* expression during the stage of latent viral infection, with or without cycloheximide treatments, suggesting that *hhi1* expression is regulated by different mechanisms during latent and productive viral infection stages.

***hhi1* and *pag1* behave differently in gene expression and RNA stability.** In order to elucidate the stabilities of *hhi1* and *pag1* transcripts, infected cells were treated with actinomycin D at a concentration of 10 μg/ml, and the transcripts of *hhi1* and *pag1* were analyzed by Northern hybridizations. Actinomycin D can suppress transcription, enabling measurement of the stability of transcripts already present at the time of drug addition. When actinomycin D was added to the medium 1 h before infection with *Hz*NV-1 virus, neither *hhi1* nor *pag1* transcripts could be detected in total cellular RNA (Fig. 3, lanes 2). Since both *hhi1* and *pag1* were early genes and their transcripts were expressed right after viral infection, the stability of their transcripts could therefore be evaluated by adding actinomycin D at 0.5 hpi to the culture medium. Northern blot analysis showed that the level of *hhi1* transcripts had decreased by up to 95% by 12 hpi (Fig. 3A), while the level of *pag1* transcripts had decreased by only ~28% over the same period of time (Fig. 3B). These data suggest that *pag1* transcript is more stable than *hhi1* transcript.

To investigate whether host factors are sufficient to turn on *hhi1* expression, the plasmid pGL3-*hhi1* was constructed in which the coding region of luciferase gene was placed under

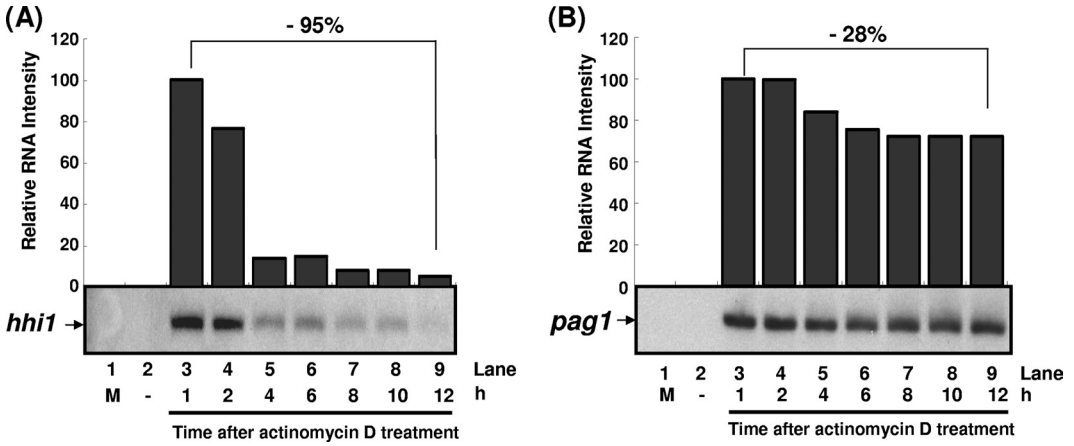

FIG. 3. Stability analysis of *hhi1* and *pag1* transcripts upon actinomycin D treatment. The stabilities of *hhi1* (A) and *pag1* (B) transcripts were analyzed in infected cells treated with actinomycin D. Lane 1, mock-infected cells. In lanes 2 cells were infected with virus 1 h after actinomycin D treatment, and total RNA was harvested at 1 hpi. In lanes 3 to 9, cells were infected with virus 0.5 h prior to actinomycin D treatment, and total RNA was then extracted at the indicated time after actinomycin D addition for the detection of *hhi1* and *pag1* transcripts.

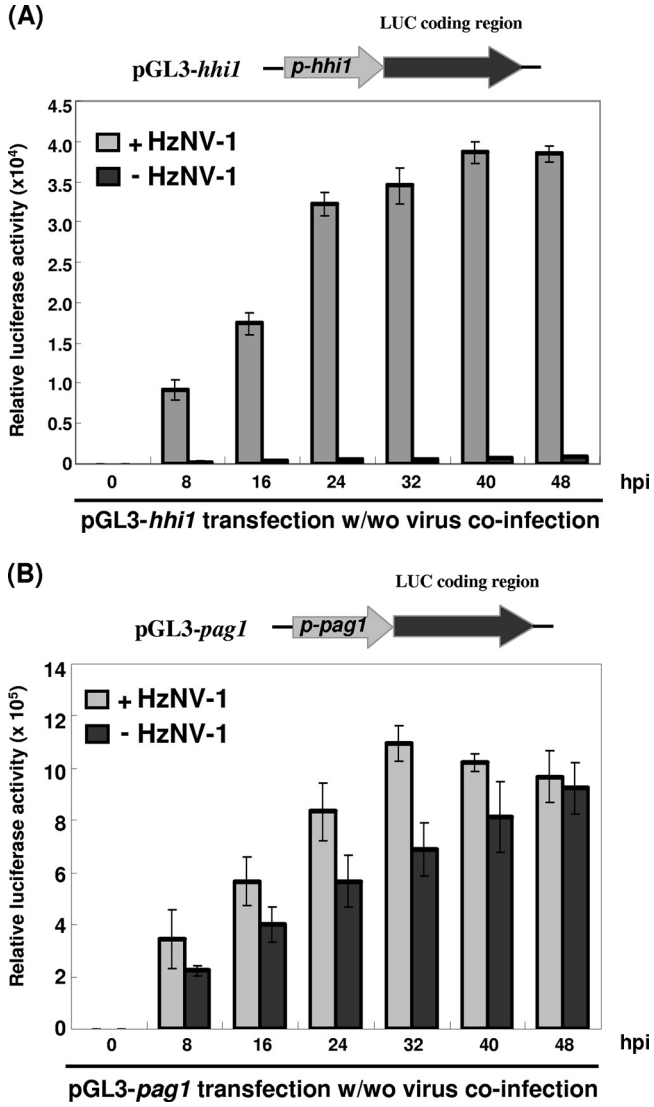

FIG. 4. Transient expression of *hhi1* and *pag1* promoters in Sf-21 cells. Luciferase activities driven by *hhi1* (A) or *pag1* (B) promoters with or without (w/wo) *Hz*NV-1 virus infections were assayed at 8, 16, 24, 32, 40, and 48 hpi.

the control of the *hhi1* promoter (31). pGL3-*hhi1* was transfected into Sf-21 cells with or without *Hz*NV-1 viral infection. No luciferase activity could be detected in cells transfected with pGL3-*hhi1*, suggesting that the *hhi1* promoter is not active or functions weakly in the absence of viral infection. In this experiment, *hhi1* promoter was stimulated upon *Hz*NV-1 virus infection (Fig. 4A), indicating that the *hhi1* promoter could be activated by *Hz*NV-1 virus infection or transactivated by viral gene products. Since it is known that host factors are sufficient to drive the expression of the early gene *pag1* (4), a similar experiment was conducted to compare the promoter activity of *pag1* with that of *hhi1*. Plasmid pGL3-*pag1* containing the full-length *pag1* promoter region (−727 to +29) to drive the expression of luciferase was constructed previously (4). The *pag1* promoter activity was tested in the presence and absence of *Hz*NV-1 virus infection in Sf-21 cells. The *pag1* promoter could be activated with or without the coinfection of *Hz*NV-1 virus (Fig. 4B), while viral factor(s) were clearly needed for the optimal expression of *hhi1* promoter.

***hhi1* stimulates viral reactivation from latent cells.** The *hhi1* gene was expressed at the very early stage during productive *Hz*NV-1 virus infection but was not detectable during latent *Hz*NV-1 virus infection (3). Thus, it is interesting to study whether *hhi1* has any function during latent *Hz*NV-1 virus infection. Two plasmids, pKShE and pKShH1 (Fig. 5A), which express enhanced green fluorescent protein (GFP) and the *hhi1* gene product, respectively, under the control of heat shock 70 promoter, were transfected into latently infected SFP4 cells. At 12 hpi with the transfection of plasmid pKShH1, most of the latently infected Sf-21 cells were lysed (Fig. 5B). Supernatants of the transfected cells were harvested, and virus titers were determined. A significantly higher titer of the virus was detected in the *hhi1*-transfected cells, for which the titer was 127 times greater than that in the pKShE-transfected cells. Furthermore, similar, low titers of viruses were detected in the medium of the latently infected Sf-21 cells with or without the transfection of control plasmid pKShE. These results suggest that the expression of HHI1 can switch *Hz*NV-1 virus infection from a latent to productive viral infection cycle (Fig. 5C).

***hhi1* transfection can activate many early genes in latently infected cells.** We have shown that *hhi1* expression can activate *Hz*NV-1 virus in latently infected Sf-21 cells. Next, *Hz*NV-1 virus early genes that may function in viral DNA transcription (*orf60*, *orf75*, *orf90*, *orf101*, and *orf121*) or viral gene replication (*orf131*), based on their sequence homology to cognate AcMNPV early genes known to function for transcription or DNA replication (28), were tested to see whether they are turned on as a result of entering into the productive cycle of viral infection. The coding region of *hhi1* was transfected into latently infected SFP4 cells, and expression of early genes was monitored by RT-PCR. Signals were detected for all the tested genes upon viral reactivation, suggesting that the transient expression of *hhi1* turned on the expression of all these early *Hz*NV-1 viral genes either directly or indirectly, and they may eventually contribute to viral reactivation from latency (Fig. 6).

To further confirm the RT-PCR and qPCR results and to determine whether HHI1 activates the expression of these genes directly, the promoter regions of these genes were cloned into plasmid pGL3. Promoter activities were then determined with or without cotransfection of the *hhi1* construct (Fig. 7A). Expression of *hhi1* could directly stimulate the promoter of both *orf90* and *orf121*, whereas the promoter activities of the other early genes tested could not be stimulated by expression of *hhi1* alone. The other early genes became active only upon virus infection, suggesting that their activation requires the assistance of viral gene products (Fig. 7B).

To investigate whether *hhi1* is solely responsible for switching the virus from latent infection to productive infection, we constructed expression vectors for several of the aforementioned early genes of *Hz*NV-1. Latently infected cells were transfected with these expression vectors, and the supernatants of the transfected cells were harvested at 48 hpt for virus titer analysis. Our results revealed that virus titer was increased by a magnitude of 2 to 3 logs when latently infected cells were transfected with the *hhi1*-expressing plasmid (Fig. 7C). This

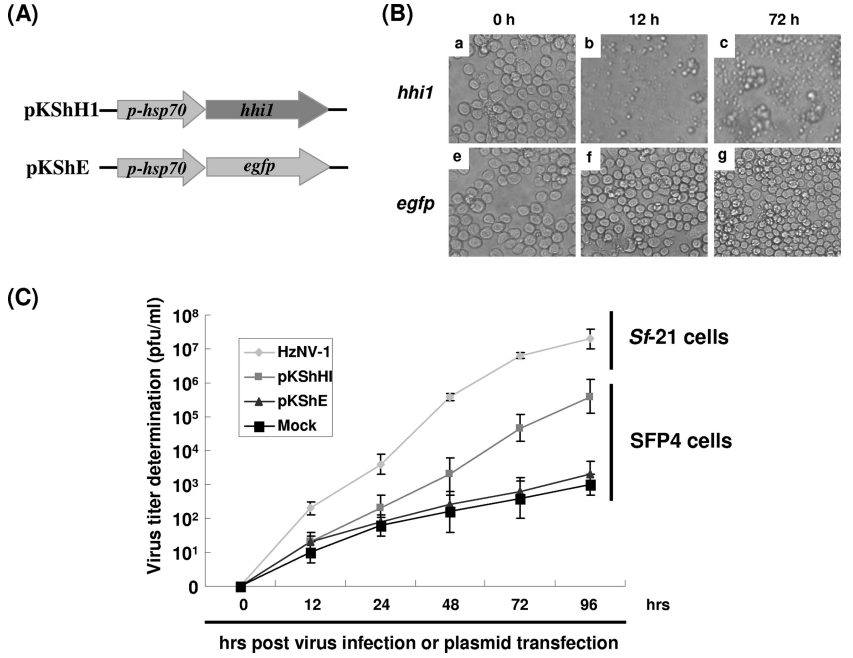

FIG. 5. Induction of viral reactivation by the *hhi1* gene in latent cells. (A) The map of plasmids pKShH1 and pKShE. In these two plasmids, *hhi1* and *egfp* coding regions were driven by the heat shock promoter. (B) Change of cell morphologies over time after plasmid transfection. Latently infected SFP4 cells were transfected with plasmids pKShH1 (a to c) and pKShE (e to g), and cell images were taken at different time points posttransfection. (C) Viral titers estimated from productively and latently infected cells. Latently infected cells were transfected with plasmids pKShH1 and pKShE separately, and the titers of the released viruses were determined at different time points. Sf-21 cells were infected with wild-type virus (*Hz*NV-1) as a control.

result indicates that *hhi1* plays a key role in virus reactivation from latency although both *orf60* and *orf90* also gave low levels of viral reactivation.

Establishment of latent cells by *hhi1* knockdown. In this report, we found that overexpression of *hhi1* in latent cells could stimulate viral DNA accumulation and reactivate cells from the latent to the productive infection stages. We therefore further investigated whether latent infection could be promoted when *hhi1* expression was suppressed during productive infection. Sf-21 cells were first transfected with siRNA targeting the *hhi1* transcripts, followed by *Hz*NV-1 infection at an MOI of 1. RT-PCR confirmed that suppression of *hhi1* expression by siRNA was successful (Fig. 8A). The number of colonies was then recorded at 12 days postinfection. Most of the Sf-21 cells died when they were infected with *Hz*NV-1 virus alone, and only a small percentage of cells became latently infected (Fig. 8B). The percentage of latently infected cells did not increase in cells transfected with siRNA targeting the *egfp* transcript, which served as a control. However, the number of latently infected cell clones increased drastically when *hhi1* expression was suppressed by siRNA (Fig. 8C). We also analyzed gene expression of *pag1* and *hhi1* in *Hz*NV-1-infected cells at 12 days postinfection with or without *hhi1* knockdown (Fig. 8D). *hhi1* expression could be detected only in cells productively infected with *Hz*NV-1 (Fig. 8D, lane 1), whereas *pag 1* expression could be detected in productive, SFP4, and *hhi1* knockdown-induced latent cells (Fig. 8D, lanes 1, 3 and 4, respectively). These results indicate that latent viral infection correlates with the absence of *hhi1* expression.

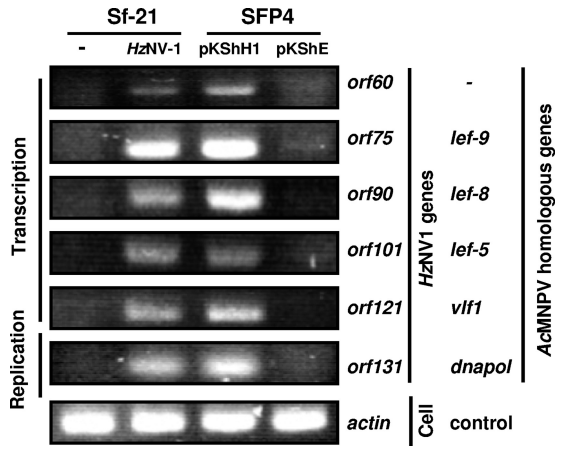

FIG. 6. Analysis of *Hz*NV-1 viral early transcripts which are induced by *hhi1* transfection. Sf-21 and latently infected cells (SFP4) were transfected with plasmid pKShH1 (expresses HHI1) or pKShE (expresses enhanced GFP), and the expression of tested early *Hz*NV-1 transcripts was analyzed by RT-PCR using primers. The early transcripts derived from productive *Hz*NV-1 virus infection of the Sf-21 cells were used as controls; the expression of actin transcript served as another control. These early genes were categorized into two groups based on their predicted functions in either viral DNA replication or viral gene transcription (28).

DISCUSSION

Previously, we found that during latent viral infection, PAT1 is the only detectable viral transcript while the *hhi1* transcript,

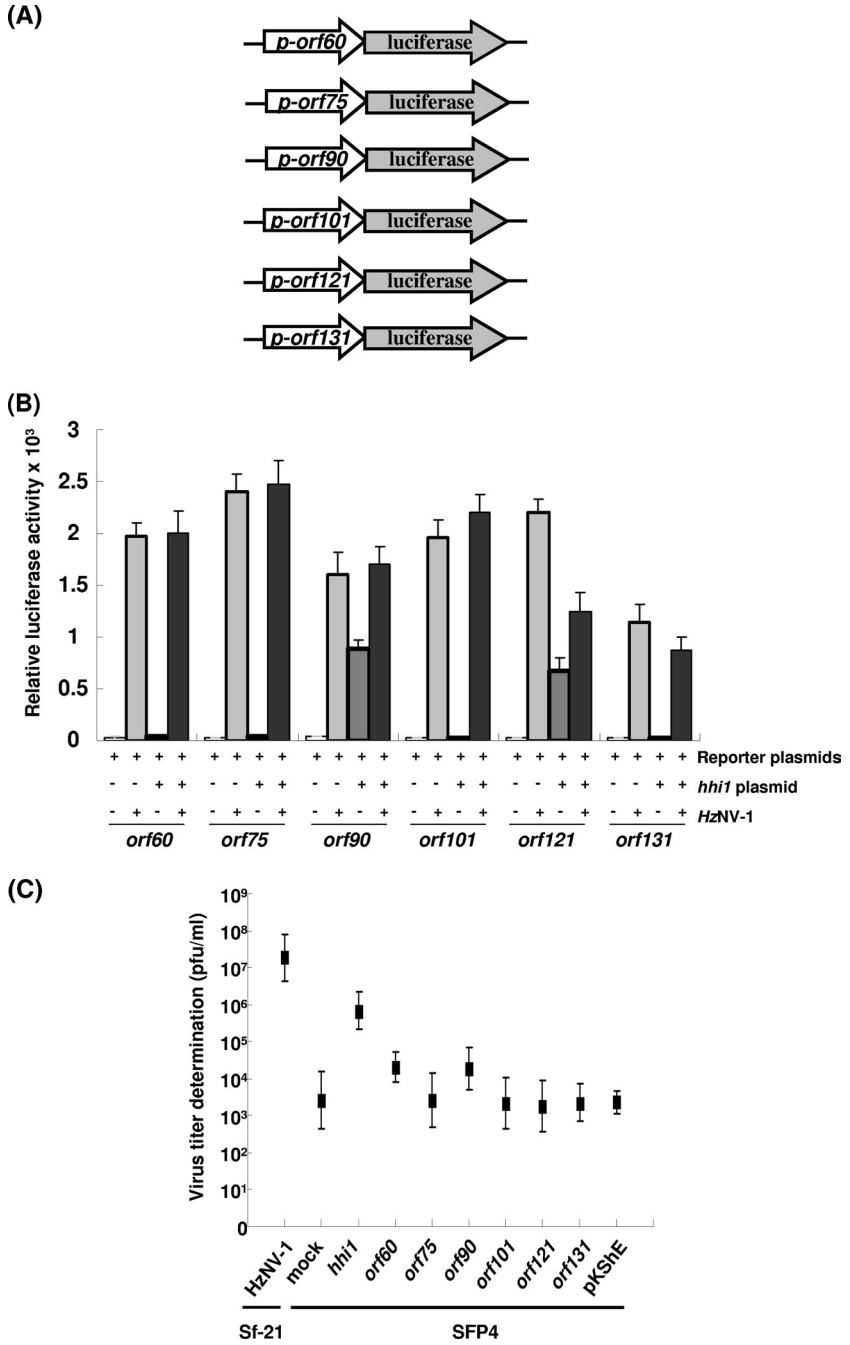

FIG. 7. Activation analysis of viral early promoters by the *hhi1* gene. (A) A list of early promoters for activation assays using luciferase as reporters. (B) The analysis of *hhi1* and *Hz*NV-1 in the activation of different viral early promoters. Luciferase activities driven by different promoter constructs were assayed by coinfection of *Hz*NV-1 virus or cotransfected with *hhi1* construct (Fig. 6A) in Sf-21 cells. (C) Virus titers estimated from latently infected cells. Latently infected cells were transfected with plasmids expressing several viral early genes, and virus titers were determined at 72 hpt. Sf-21 cells were also infected with *Hz*NV-1 as a control.

although not detectable during latent viral infection, was one of the very early transcripts expressed during productive viral infection (3). During productive infection, *hhi1* transcript was found to initiate early and then diminish at late viral infection stages. This reduction in *hhi1* transcript might be the result of feedback inhibition from *hhi1's* own gene products or of the functions of other viral gene products which either block the transcription of *hhi1* or degrade *hhi1* transcript. Since blocking cellular translation with cycloheximide increases the accumulation of *hhi1* transcript, it is possible that the presence of certain host or viral factors may negatively regulate *hhi1* expression.

Although during productive viral infection several late transcripts other than *hhi1* were also expressed by using a probe

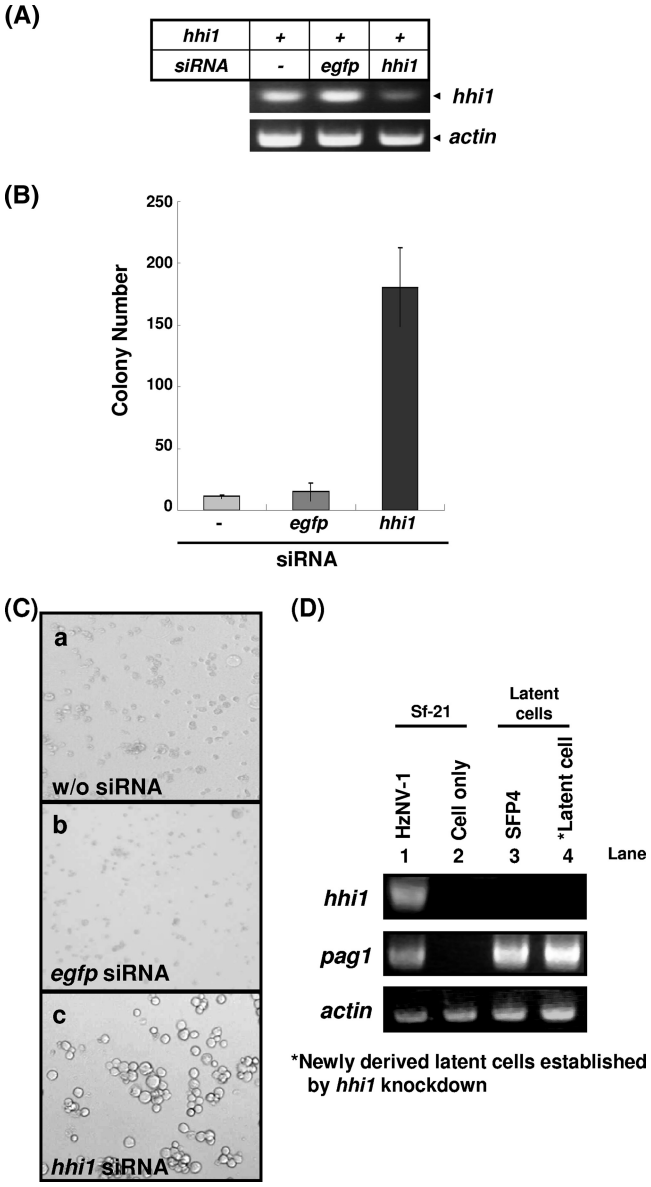

FIG. 8. Establishment of latent viral infection by *hhi1* knockdown. (A) RT-PCR showed that siRNA can efficiently suppress *hhi1* expression in *Hz*NV-1-infected cells at 12 hpi. (B) Generation of latently infected colonies by *Hz*NV-1 virus infection in siRNA-transfected cells. Sf-21 cells (4×10^4) were transiently transfected with siRNA (against *hhi1* or *egfp*) followed by *Hz*NV-1 infection. The numbers of surviving cell colonies were calculated at 12 days postinfection. Data (means ± standard deviations) were collected from triplicate assays. (C) Sf-21 cells were infected with *Hz*NV-1, and the images were taken at 12 days postinfection: frame a, Sf-21 cells infected with *Hz*NV-1 only; frame b, Sf-21 cells transfected with *egfp* siRNA and subsequently infected with *Hz*NV-1; frame c, Sf-21 cells transfected with *hhi1* siRNA and subsequently infected with *Hz*NV-1. (D) Confirmation of *hhi1* and *pag1* expression in cells by RT-PCR. Cells in lane 1 were infected with *Hz*NV-1, and RT-PCR was performed at 2 hpi. Lane 2, cells only; lane 3, latent SFP4 cells; lane 4, *hhi1* knockdown-induced latently infected cells (for lane 4, RT-PCR was performed at 20 days postinfection).

covering the HindIII-I fragment (data not shown), these late transcripts were no longer detectable after treatment with a protein synthesis inhibitor, cycloheximide. In this work, cycloheximide had a positive effect on the accumulation of *hhi1* transcript, indicating that de novo proteins synthesis is not required for *hhi1* gene expression. It should be noted, however, that in latently infected cells, no *hhi1* expression was detected with or without cycloheximide treatment. This implies that the expression of *hhi1* is regulated by different mechanisms during the latent and productive phases of infection.

In our transient expression assay, we found that without viral coinfection, the expression level of *hhi1* promoter is relatively low. To study whether *hhi1* expression had any effect on latently infected cells, heat shock promoter (15) was used (by plasmid pKShH1) to drive the coding region of the *hhi1* gene. In this experiment, we found that transfection of this plasmid into latent cells resulted in viral reactivation and increased viral DNA replication (data not shown), suggesting that *hhi1* is the gene responsible, or at least one of the genes responsible, for viral reactivation. Also, although *hhi1* functions in viral reactivation, some other genes may be required to further assist the reactivation of the virus from the latent state. Certainly, further studies are needed to investigate all of these possibilities.

Previously, we showed that during latent viral infection, spontaneous viral reactivation occurs in some 0.2% of the cells, which results in the lysis of the host cells and contributes to low background viral titers of an order of 10^3 in the medium (19). It would be interesting to know the factor(s) and mechanism(s) in individual cells that determine whether viruses remain latent or reactivate. It is possible that host factors may block the *hhi1* promoter and suppress its expression. In this case, the virus will remain latent; on the other hand, if the suppression of host factors is not strong enough, perhaps due to poor nutritional conditions, aging of the host cells, or other as yet unknown factors, viral reactivation may result. Alternatively, host factors, or even viral factors that can stimulate the expression of *hhi1*, may appear by an as-yet-unknown mechanism and turn on *hhi1* for viral reactivation.

Upon *hhi1* stimulation, the latent cells release viruses. Although the virus titer in these cells is significantly higher than that from originally latently infected cells or pKShE-transfected cells, it is not as high as that observed during productive viral infection. This may be because the latent cells contain a very high proportion of concatemeric or defective viral genomes (19). Thus, after reactivation, these viral genomes may be too big to be enclosed into virus particles. Alternatively, even if they can be packed as viral particles, due to the nature of genome deletion, most of them may become defective interference viral particles and interfere with subsequent titer determination.

Latent baculovirus infections are reported frequently; however, they can be observed only by spontaneous activation or by the detection of trace amounts of viral transcripts or genome in cell lines or insect populations (2, 12, 13). So far, the genes responsible for latent establishment and viral reactivation are largely unknown. Herpes simplex viruses (HSV) can undergo productive infection in epithelial cells and latent infection in sensory neurons. During latency, the virus persists until reactivation, leading to recurrent productive infection and transmission to new hosts (17). The viral immediate-early protein ICP0 of HSV-1 is one of the very few gene products known to cause viral reactivation from latency. ICP0 is a multifunctional gene product which is capable of regulating viral and host

cellular gene expression at transcriptional, translational, and posttranslational levels (7, 9). Although there is no significant sequence homology between ICP0 and *hhi1*, our experiments showed that *hhi1* is one of these novel genes that functions in viral reactivation.

HSV-1 and *Hz*NV-1 are quite different viruses and belong to distinct virus families without obvious similarity in genomic sequence or morphology of viral particles (27). The hosts of these viruses are also from different phyla. Thus, their mechanisms for reactivation might be quite different. However, HSV-1 has striking similarities in life cycle with *Hz*NV-1 virus. During productive viral infection, HSV-1 also gives rise to more than 100 transcripts, and during latent viral infection, only one gene, the latency-associate gene 1 (LAT1) (29) is expressed. Interestingly, LAT1 may function similarly to PAT1 since both produce stable noncoding RNAs (4). With such differences in origin, the possibility that they may use similar mechanisms for viral reactivation is even more intriguing. So far, very few genes are known to be involved in viral reactivation in mammals, and *hhi1* is currently the only gene known to have a function in viral reactivation in insects.

In our experiments, some viral early genes, which may be involved in either viral DNA replication or viral gene transcription or both, were compared in productive, latent, and reactivated *Hz*NV-1-infected cells. Our results showed that all the tested early genes of *Hz*NV-1 were expressed in the reactivated cells. This suggests that *hhi1* can directly or indirectly activate the expression of many early genes, and then viral DNA replication and, perhaps, the transcription of many other genes are turned on. Eventually, the expression of *hhi1* leads to the reactivation of *Hz*NV-1 virus from latency. Possible functions of these early genes were predicted by their sequence homology to the cogent genes from AcMNPV (5); however, their actual functions are not yet clear and still await further experiments to elucidate their roles in the life cycle of *Hz*NV-1 virus infection in host cells.

The identification of *hhi1* as the gene, or one of the genes, responsible for viral reactivation provides a key for further mechanistic study of viral reactivation. It would be interesting to learn how *hhi1* is regulated during productive and latent viral infections. Any interactions of virus and host factors required for *hhi1* gene functioning or environmental changes which eventually lead to viral reactivation during latent viral infection are important issues for further study of this fascinating viral infection cycle.

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REFERENCES

1. **Burand, J. P., B. Stiles, and H. A. Wood.** 1983. Structural and intracellular proteins of the nonoccluded baculovirus Hz-1. J. Virol. **46:**137–142.
2. **Burden, J. P., R. D. Possee, S. M. Sait, L. A. King, and R. S. Hails.** 2006. Phenotypic and genotypic characterisation of persistent baculovirus infections in populations of the cabbage moth (*Mamestra brassicae*) within the British Isles. Arch. Virol. **151:**635–649.
3. **Chao, Y. C., H. A. Wood, C. Y. Chang, H. J. Lee, W. C. Shen, and H. T. Lee.** 1992. Differential expression of Hz-1 baculovirus genes during productive and persistent viral infections. J. Virol. **66:**1442–1448.
4. **Chao, Y. C., S. T. Lee, M. C. Chang, H. H. Chen, S. S. Chen, T. Y. Wu, F. H. Liu, E. L. Hsu, and R. F. Hou.** 1998. A 2.9-kilobase noncoding nuclear RNA functions in the establishment of persistent Hz-1 viral infection. J. Virol. **72:**2233–2245.
5. **Cheng, C. H., S. M. Liu, T. Y. Chow, Y. Y. Hsiao, D. P. Wang, J. J. Huang, and H. H. Chen.** 2002. Analysis of the complete genome sequence of the Hz-1 virus suggests that it is related to members of the *Baculoviridae*. J. Virol. **76:**9024–9035.
6. **Cliffe, A. R., and D. M. Knipe.** 2008. Herpes simplex virus ICP0 promotes both histone removal and acetylation on viral DNA during lytic infection. J. Virol. **24:**12030–12038.
7. **Everett, R. D.** 2000. ICP0, a regulator of herpes simplex virus during lytic and latent infection. Bioessays **22:**761–770.
8. **Granados, R. R.** 1978. Early events in the infection of *Heliothis zea* midgut cells by a baculovirus. Virology **90:**170–174.
9. **Hagglund, R., and B. Roizman.** 2004. Role of ICP0 in the strategy of conquest of the host cell by herpes simplex virus 1. J. Virol. **78:**2169–2178.
10. **Hink, W., and C. M. Ignoffo.** 1970. Establishment of a new cell line (IMC-*HZ*-1) from ovaries of cotton bollworm moths, *Heliothis zea* (Boddie). Exp. Cell Res. **60:**307–309.
11. **Huang, Y. S., M. Hedberg, and C. Y. Kawanishi.** 1982. Characterization of the DNA of a nonoccluded baculovirus, Hz-1V. J. Virol. **43:**174–181.
12. **Hughes, D. S., R. D. Possee, and L. A. King.** 1993. Activation and detection of a latent baculovirus resembling Mamestra brassicae nuclear polyhedrosis virus in *M. brassicae* insects. Virology **194:**608–615.
13. **Hughes, D. S., R. D. Possee, and L. A. King.** 1997. Evidence for the presence of a low-level, persistent baculovirus infection of *Mamestra brassicae* insects. J. Gen. Virol. **78:**1801–1805.
14. **Ignoffo, C. M., M. Shapiro, and W. F. Hink.** 1971. Replication and serial passage of infectious *Heliothis* nucleopolyhedrosis virus in an established line of *Heliothis zea* cells. J. Invertebr. Pathol. **18:**131–134.
15. **Ingolia, T. D., E. A. Craig, and B. J. McCarthy.** 1980. Sequence of three copies of the gene for the major *Drosophila* heat shock induced protein and their flanking regions. Cell **21:**669–679.
16. **Kelly, D. C., T. Lescott, M. D. Ayres, D. Carey, A. Coutts, and K. A. Harrap.** 1981. Induction of a nonoccluded baculovirus persistently infecting *Heliothis zea* cells by *Heliothis armigera* and *Trichoplusia ni* nuclear polyhedrosis viruses. Virology **112:**174–189.
17. **Kent, J. R., and N. W. Fraser.** 2005. The cellular response to herpes simplex virus type 1 (HSV-1) during latency and reactivation. J. Neurovirol. **11:**376–383.
18. **Lee, J. C., H. H. Chen, H. L. Wei, and Y. C. Chao.** 1993. Superinfection-induced apoptosis and its correlation with the reduction of viral progeny in cells persistently infected with Hz-1 baculovirus. J. Virol. **67:**6989–6994.
19. **Lin, C. L., J. C. Lee, S. S. Chen, H. A. Wood, M. L. Li, C. F. Li, and Y. C. Chao.** 1999. Persistent Hz-1 virus infection in insect cells: evidence for insertion of viral DNA into host chromosomes and viral infection in a latent status. J. Virol. **73:**128–139.
20. **Lo, H. R., and Y. C. Chao.** 2004. Rapid titer determination of baculovirus by quantitative real-time polymerase chain reaction. Biotechnol. Prog. **20:**354–360.
21. **McIntosh, A. H., and C. M. Ignoffo.** 1981. Establishment of a persistent baculovirus infection in a lepidopteran cell line. J. Invertebr. Pathol. **8:**395–403.
22. **McIntosh, A. H., J. J. Grasela, and C. M. Ignoffo.** 2007. *In vitro* host range of the Hz-1 nonoccluded virus in insect cell lines. In Vitro Cell Dev. Biol. Anim. **43:**196–201.
23. **O'Reilly, D. R., L. K. Miller, and V. A. Luckow.** 1994. Baculovirus expression vectors: a laboratory manual. Oxford University Press, New York, NY.
24. **Podgwaite, J. D., and H. M. Mazzone.** 1986. Latency of insect viruses. Adv. Virus Res. **31:**293–320.
25. **Ralston, A. L., Y. Huang, and C. K. Kawanishi.** 1981. Cell culture studies with the IMC-Hz-1 nonoccluded virus. Virology **115:**33–44.
26. **Wang, R. F., and S. R. Kushner.** 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. Gene **100:**195–199.
27. **Wang, Y., R. G. Kleespies, A. M. Huger, and J. A. Jehle.** 2007. The genome of *Gryllus bimaculatus* nudivirus indicates an ancient diversification of baculovirus-related nonoccluded nudiviruses of insects. J. Virol. **81:**5395–5406.
28. **Wang, Y., M. M. van Oers, A. M. Crawford, J. M. Vlak, and J. A. Jehle.** 2007. Genomic analysis of *Oryctes rhinoceros* virus reveals genetic relatedness to *Heliothis zea* virus 1. Arch. Virol. **152:**519–531.
29. **Whitley, R. J.** 2001. Herpes simplex viruses, p. 2461–2510. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
30. **Wood, H. A., and J. P. Burand.** 1986. Persistent and productive infections with the Hz-1 baculovirus. Curr. Top. Microbiol. Immunol. **131:**119–133.
31. **Wu, Y. L., C. Y. Liu, C. P. Wu, C. H. Wang, S. T. Lee, and Y. C. Chao.** 2008. Cooperation of *ie1* and *p35* genes in the activation of baculovirus AcMNPV and *Hz*NV-1 promoters. Virus Res. **135:**247–254.
32. **Wu, C. P., J. Y. Wang, T. Y. Huang, H. R. Lo, and Y. C. Chao.** 2008. Identification of baculoviral factors required for the activation of enhancer-like polyhedrin upstream (*pu*) sequence. Virus Res. **138:**7–16.