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Heliothis zea Nudivirus 1 Gene *hhi1* Induces Apoptosis Which Is Blocked by the *Hz-iap2* Gene and a Noncoding Gene, *pag1*▽†

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***Heliothis zea* nudivirus 1 (*Hz*NV-1 or *Hz*-1 virus), previously regarded as a nonoccluded baculovirus, recently has been placed in the *Nudivirus* genus. This virus generates *Hz*NV-1 HindIII-I 1 (*hhi1*) and many other transcripts during productive viral infection; during latent viral infection, however, persistency-associated gene 1 (*pag1*) is the only gene expressed. In this report, we used transient expression assays to show that *hhi1* can trigger strong apoptosis in transfected cells, which can be blocked, at least partially, by the inhibitor of apoptosis genes *Autographa californica iap2 (Ac-iap2)* and *H. zea iap2* (*Hz-iap2*). In addition to these two genes, unexpectedly, *pag1*, which encodes a noncoding RNA with no detectable protein product, was found to efficiently suppress *hhi1*-induced apoptosis. The assay of pro-*Sf*-caspase-1 processing by *hhi1* transfection did not detect the small P12 subunit at any of the time intervals tested, suggesting that *hhi1* of *Hz*NV-1 induces apoptosis through alternative caspase pathways.**

Heliothis zea nudivirus 1 (*Hz*NV-1 or *Hz*-1 virus) is an enveloped, rod-shaped, nonoccluded virus with a circular double-stranded DNA genome composed of approximately 154 open reading frames (4–8, 17, 21). This virus previously was regarded as a member of the baculovirus family, but due to its different genetic content and life cycle, it has been reclassified into a new genus of nonoccluded viruses called the *Nudivirus*. (47, 48). The *Hz*NV-1 virus has a relatively broad host range and has been reported to productively and latently infect many insect cell lines (4, 14, 24, 31, 34, 35, 40). Previously, we found that more than 100 transcripts are produced during productive *Hz*NV-1 virus infection (6); during this phase of infection, a gene located on the *Hz*NV-1 HindIII-I fragment (*hhi1*) was found to generate an abundant 6.2-kb transcript within 0.5 h postinfection (hpi) (50, 51). In contrast to the expression pattern of many early genes of baculovirus, the expression of *hhi1* requires the assistance of *Hz*NV-1 viral infection (51). In another experiment, we showed that *hhi1* expression also can be transactivated by *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV). Such transactivation is due to the function of *ie1* and *p35* genes of this heterologous virus (50). We have reported before that *hhi1* can serve as a transcriptional activator and is involved in viral reactivation from latently infected cells (50, 51). Also, during latent viral infection, only one transcript derived from persistency-associated gene 1 (*pag1*) was detectable. *pag1* gives rise to a unique noncoding RNA, named persistency-associated transcript 1 (PAT1), which was found to be responsible for, or at least involved in, the establishment of latent viral infection (5, 6).

*Hz*NV-1 virus infection of host Sf-21 cells results in the necrosis of most of the infected cells and produces high yields of virus progeny (28, 29), whereas the superinfection of *Hz*NV-1 latently infected Sf-21 cells induces quick apoptosis and yields relatively lower levels of virus progeny (28, 29). Previous experiments showed that viral antigen could be detected in necrotic cells but not in apoptotic cells (29). Thus, apoptosis seems to serve as a part of the host's defense mechanism against successful virus infection in insect cells latently infected with *Hz*NV-1 virus. However, the viral gene(s) involved and the mechanism for the induction of apoptosis in cells infected with *Hz*NV-1 virus are not yet clear.

Caspases are a group of cysteine proteases that are the central component of apoptosis machinery. These proteases also were identified in insects. *Sf*-caspase-1 is the main effector caspase of Sf cells (32). To overcome the antiviral defense response of the host (i.e., apoptosis) and thus to ensure the viral infection process, baculoviruses express two types of genes known to suppress cell death: *p35* and inhibitors of apoptosis (*iap* family) (1, 9, 49). P35 is a stoichiometric inhibitor of caspases and can function in phylogenetically disparate organisms to block apoptosis induced by a variety of signals (16, 28, 43). The IAP family is another group of apoptosis inhibitors. Some of them have been reported to be possible substitutes for P35 to block apoptosis during the infection of baculovirus *Ac*MNPV in insect cells (44). Most IAP proteins contain a specific motif called the baculovirus IAP repeat (BIR) which is responsible for mediating apoptosis suppression. Currently, the baculovirus *iap* genes are thought to act as a sink for IAP antagonists and thereby inhibit apoptosis (3, 9).

In this report, we further demonstrated that the early gene *hhi1* of *Hz*NV-1 possesses the ability to directly or indirectly

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TABLE 1. Primer sequences used in this study

Primer name[a]	Primer sequence (5′–3′)
hhi1 F	ATTCCCGGGCTCTCCTCTACAATCATGTCTAC CGTG
hhi1 R	ATTCCCGGGCTCAGATTCACAGTATGGTTCACG
p35 F	ATTGAATTCACCATTGCAAAATGTGTGTA
p35 R	ATTCTGCAGTTTTAACATTTATTTAATTG
Ac-iap1 F	ATTGAATTCTTTAACGAGCTAAAATGAACG
Ac-iap1 R	ATTCTTACACCACATTTATGCAGCACCAT
Ac-iap2 F	ATTGAATTCGGAATAAACTATAAAATGAA
Ac-iap2 R	ATTCTGCAGAATGTTTACTGAGGTAATGT
Hz-iap1 F	ATTGAATTCCAGCGAGATGAATGAAATTGA
Hz-iap1 R	ATTCTGCAGCACTAAATATTTAAACAACAA
Hz-iap2 F	ATTGAATTCTATCGTAAAATGTCGACCGC
Hz-iap2 R	ATTCTGCAGATAATTATATTTAAAGTAAAT
Sf-cas1 F	GCGGATCCATACTGGACGGAAAACAA
Sf-cas1 R	GCGAATTCTCTGTGGGACTGCTTCTT
ie1 F	GCCCATGGGTACGCAAATTAATTTTAAC
ie1 R	GCCTCGAGATTAAATTCGAATTTTTTATA

[a] F, forward; R, reverse.

promote apoptosis in host cells. We also found that although *p35* can prevent *ie1*-induced apoptosis in Sf-21 cells, it was unable to prevent *hhi1*-induced apoptosis. In contrast, *Ac*MNPV *iap2*, which has not previously been found to function as an inhibitor of apoptosis in lepidopteran cells, was able to suppress *hhi1*-induced apoptosis in Sf-21 cells. In addition, we discovered that the noncoding RNA PAT1, which was found to function in the establishment of latent viral infection, could suppress *hhi1*-induced apoptosis. The mechanism underlying this apoptosis suppression was analyzed, and it was found that apoptosis induced by *hhi1* can be blocked by two totally different mechanisms, including the functions of PAT1 and IAP.

MATERIALS AND METHODS

Cells and virus. *Spodoptera frugiperda* IPLB-Sf-21 was incubated in TC-100 insect cell culture medium containing 10% fetal bovine serum (FBS) at 26°C (Gibco BRL) (38, 50, 51). Standard *Hz*NV-l virus was derived by a serial dilution of the stock viral solution and isolated by plaque purification. The titers of the virus clones were estimated by both quantitative PCR (qPCR) (33) and 50% tissue culture infective doses ($TCID_{50}$) (38).

Plasmid construction. Plasmid pKSh was constructed by inserting the *hsp 70* promoter (*p-hsp*) (11, 22) into plasmid pBluescript II KS(−) (Stratagene). Unless otherwise stated, His tags were added to the coding regions of *egfp*, *hhi1*, *p35*, *Autographa californica iap1 (Ac-iap1)*, *Ac-iap2*, *H. zea iap1* (*Hz-iap1*), *Hz-iap2*, *Sf*-caspase-1, and *ie1* by PCR (Table 1), and the PCR fragments were inserted downstream of plasmid pKSh to generate the expression plasmids pKShE (*egfp*), pKShH1 (*hhi1*), pKShp35 (*p35*), pKShAiap1 (*Ac-iap1*), pKShAiap2 (*Ac-iap2*), pKShHiap1 (*Hz-iap1*), pKShHiap2 (*Hz-iap2*), pKShC1 (*Sf*-caspase-1), and pKShIE (*ie1*), respectively. Plasmids pKShH1ΔH, pKShHiap2ΔH, and pKShIEΔH, which express untagged HHI1, IAP2, and IE1, also were constructed. These plasmids function either to induce apoptosis (HHI1 and IE1) or to block *hhi1*-induced apoptosis (IAP2) in Sf-21 cells with levels undistinguishable from that induced by their counterplasmids without His tags (data not shown). The *Hz*NV-1 viral early promoter regions of *pag1* (+29 to ~727) (51) were obtained by PCR using *Hz*NV-1 viral DNA as a template. Promoter regions were ligated into pGL3-basic vector (Promega) to obtain plasmid pGLpL for activity assay with or without pKShp35 transfection. To assay the suppression of *hhi1*-induced apoptosis by *pag1*, we obtained the *pag1* coding region by PCR (51), and the PCR product was inserted downstream of the *pag1* promoter (*p-pag1*) (5) of plasmid pKSp to generate the expression plasmid pKSpP1. All of the insertions of newly constructed plasmids were confirmed by sequencing.

DNA transfection into cells. A total of 2×10^5 cells per well were seeded into a 24-well culture plate (Corning) and then transfected with 0.5 μg of appropriate plasmid DNAs using Cellfectin (Invitrogen) according to the manufacturer's protocol (Gibco BRL). For the apoptosis assay and *hhi1* expression level assay, plasmids expressing *hhi1* coding sequence were cotransfected with pKShE, pKSpP1, pKShH1, pKShC1, pKShAiap1, pKShAiap2, pKShHiap1, pKShHiap2, or pKShIE. The nucleotide sequences of the *hhi1* and *pag1* genes used in these experiments were submitted to GenBank, and the accession numbers assigned were AF264019 (*hhi1*) and NC004156 (*pag1*), respectively (51).

Annexin V-FITC staining and viability assay. Cells were collected at different time points, washed with phosphate-buffered saline (PBS), and stained with fluorescein isothiocyanate (FITC)-conjugated annexin V (BD Biosciences) for 20 min at room temperature in the dark. The stained cells then were analyzed by flow cytometry (Beckman Coulter). Apoptotic cells were identified as those with annexin V-FITC staining only, and the results were expressed as the proportion of such cells among the total number of cells analyzed. Upon the transfection of Sf-21 cells with the *hhi1* construct, cells were stained with calcein AM (Live/Dead Viability kit; Molecular Probes) to quantify the effect of *hhi1* on cell viability. Data (means ± standard deviations) were collected from triplicate assays of three independent experiments.

Caspase activity assay of apoptotic cells. *hhi1*-transfected cells were lysed at different time points posttransfection (hpt) and stained with fluorogenic substrates specific for different caspases immobilized in the wells of 96-well plates (ApoAlert caspase assay plates; Clontech). At the same time, untransfected cells were exposed to UV light (50 mJ/cm^2 for 30 min) to induce apoptosis in these cells as a control.

TUNEL assay of apoptotic cells. For terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assays, Sf-21 cells (4×10^5/well) were seeded in a 24-well culture plate (Corning, Acton, MA) and then left untransfected or transfected with *hhi1* DNA using Cellfectin (Invitrogen, Carlsbad, CA). We then tested the apoptosis inhibitors DEVD-CHO and zVAD-fmk (BD Biosciences), using concentrations ranging from 2 to 12 μM, and found that 10 to 12 μM gave the best results for both inhibitors; therefore, 10 μM was selected for further assays. Cells were collected at different time points after treatments, fixed with 4% methanol-free formaldehyde, and stained with fluorescein-12-dUTP (Promega) for 60 min at 37°C. The stained cells then were analyzed by a fluorescence plate reader (Victor III; Perkin Elmer) using the enhanced green fluorescent protein filter set (485 nm/535 nm).

Western analyses. Sf-21 (2×10^5) cells were transfected with 1 μg of plasmids (pKSh, pKShE, pKShH1, pKShC1, pKShAiap1, pKShAiap2, pKShHiap1, pKShHiap2, pKShH1ΔH, pKShHiap2ΔH, or pKShIEΔH) using Cellfectin (Invitrogen). At 4 hpt, the transfection reagent was replaced with TC-100 medium containing 10% FBS. Transfected cells were inoculated (multiplicity of infection [MOI], 1) with *Ac*MNPV or *Hz*NV-l virus and incubated at 26°C with gentle rocking. Sf-21 cells transfected with different plasmids and/or infected with different viruses (as indicated) were suspended in 0.2 M Tris-HCl buffer containing 4% SDS, 18% glycerol, 2% β-mercaptoethanol, and 0.004% bromophenol blue. Proteins were transferred to nitrocellulose filters (Schleicher & Schuell) by electroblotting for 1 h in 200 mM glycine, 2.5 mM Tris-HCl, and 20% methanol. Filters were blocked with PBS containing 5% nonfat dried milk, 0.05% Tween 20 and incubated with primary antibodies against His tag followed by alkaline phosphatase (AP)-conjugated anti-mouse antibody (Dako).

Quantitation of viral early gene expression. RNA of transfected cells was extracted using an RNeasy Minikit (Qiagen). The RNA pellet was dissolved in 30 μl of diethyl pyrocarbonate (DEPC) water and used for cDNA synthesis. cDNA synthesis was performed using the SuperScript III first-strand synthesis system for reverse transcription-PCR (RT-PCR) by following the manufacturer's protocol (Invitrogen). *Hz*NV-1 early genes were RT-PCR amplified using the following primers: pag1-F, 5′-ACGGGAATTCAGTGTCGAGGACTT-3′; pag1-R, 5′-CATGTCTAGAACCCTACCTACCT-3′; actin-F, 5′-CGTGATGGTGGGCATGGGTCAG-3′; and actin-R, 5′-CTAATGTCACGCACGTATTCC-3′.

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

Luciferase activity assay. Luciferase assays were conducted as described previously (50). 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 against time of infection. In these experiments, luciferase activity, cellular viability, apoptosis, and caspase activity assays all were derived from triplicate assays of three independent experiments.

RESULTS

***hhi1* induces apoptosis in Sf-21 cells.** In previous experiments, we showed that *hhi1* can activate viruses in latent cells

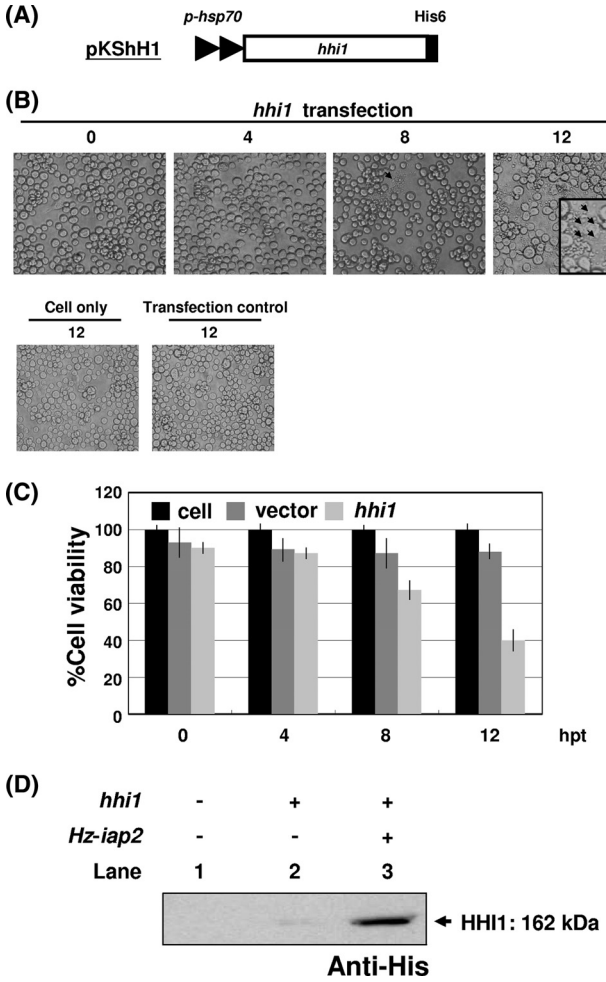

FIG. 1. Induction of apoptosis in Sf-21 cells upon transfection with or without *hhi1*. (A) Schematic of the His-tagged *hhi1*-expression vector, pKShH1, which contains an *hsp70* promoter (*p-hsp*) to drive *hhi1* expression. (B) Monolayers of Sf-21 cells were transfected with pKShH1 by Cellfectin. Cells then were examined by light microscopy and photographed at 0, 4, 8, and 12 hpt. The arrows indicate a cluster of apoptotic bodies. Healthy and pKShE-transfected (transfection control) Sf-21 cells served as negative controls. (C) Cells were stained with calcein AM at the indicated time points, and viable cells, indicated by the exclusion of the dye, were counted under a microscope. (D) Sf-21 cells were transfected with a His-tagged *hhi1* gene with or without untagged *Hz-iap2* and then analyzed by Western blotting to study the expression of HHI1. Lane 1, cells only; lane 2, cells transfected with *hhi1* only; lane 3, cells cotransfected with *hhi1* and the antiapoptotic gene *Hz-iap2*.

and be a major transactivator stimulating the expression of many early transcripts. To further characterize the function of *hhi1*, we constructed plasmid pKShH1 (51), which expressed His-tagged HHI1 (Fig. 1A). Plasmid pKShH1 was transfected into Sf-21 cells, and the transfected cells were observed under light/fluorescence microscopy. Interestingly, significant apoptosis was observed in Sf-21 cells transfected with *hhi1*-expressing vector at 8 hpt. At 12 hpt, up to 60% of the cells were found to be apoptotic bodies (Fig. 1B). Cells were stained with calcein AM (Live/Dead viability kit; Molecular Probes) to quantify the effect of *hhi1* on cell viability. In the cells transfected with *hhi1*, we found that cell viability was reduced to approximately 40 to 50% of that of the control cells transfected with the original vector. This result indicates that *hhi1* could result in more than 50% cell death of the transfected cells by 12 hpt (Fig. 1C).

To verify that HHI1 protein was properly expressed in the Sf-21 cells, plasmid pKShH1 was transfected into cells. Western analysis showed that the His-tagged HHI1 signal is weak in the transfected Sf-21 cells (Fig. 1D); this is likely due to a quick induction of cellular apoptosis by the transfection of *hhi1* (Fig. 1B). Plasmid pKShH1 then was cotransfected into Sf-21 cells with plasmid pKShHiap2ΔH, which expresses the *Hz*-IAP2 protein without a His tag. In later experiments we found that this gene, with or without the His tag, can specifically block the apoptosis induced by *hhi1*. The cotransfection of *Hz-iap2* allows us to better detect the HHI1 signal by Western blot analysis (Fig. 1D).

We further used dyes that stained early and late apoptotic markers to monitor the progression of apoptosis in host cells. First we used annexin V, which recognizes the early apoptotic marker phosphatidylserine (PS) on the plasma membrane, to observe the initiation of *hhi1*-induced apoptosis in the cells. Cells transfected with *hhi1* were stained with annexin V (annexin V-FITC apoptosis detection kit I; Clontech). By flow cytometry, we found that apoptosis signals could be detected as early as 2 h after transfection, and the intensity of the signal declined after 8 hpt (Fig. 2A and B). We also studied DNA fragmentation in the late apoptotic cells. The results of TUNEL staining (DeadEnd fluorometric TUNEL system; Clontech), which stained fragmented DNA, showed that DNA starts to fragment at about 16 h after *hhi1* transfection (Fig. 3A). As a control, cells also were treated with actinomycin D, and the treated cells were subjected to a TUNEL assay. DNA fragmentation was detected in cells treated with actinomycin D, whereas no DNA fragmentation was detected in cells not treated with actinomycin D (Fig. 3B). Based on these results, we concluded that *hhi1* could induce apoptosis in transfected cells (Fig. 3C).

Activation of caspases in *hhi1*-transfected cells. Previous reports identified a cysteine-aspartic protease (caspase) in Sf-21 cells, which has been named *Sf*-caspase-1 (1, 26, 44). When Sf-21 cells were infected with *p35* knockout baculovirus, these cells entered apoptosis through an *Sf*-caspase-1 pathway. The caspase substrate DEVD-AMC (mammalian caspase-3 substrate) can be recognized and cleaved by *Sf*-caspase-1 in Sf-21 cells infected with *p35* knockout virus (16, 20, 44). To investigate which caspase is involved in *hhi1*-induced apoptosis, we measured caspase activity in *hhi1*-transfected cells by using different substrates: VDVAD-AMC (mammalian caspase-2 substrate), DEVD-AMC (mammalian caspase-3 substrate), IETD-AMC (mammalian caspase-8 substrate), and LEHD-AMC (mammalian caspase-9 substrate) (41). These caspase substrates are cleaved predominantly by either initiator (IETD-AMC, LEHD-AMC, and VDVAD-AMC) or effector (DEVD-AMC) caspases (41). To determine whether there are differences between *hhi1*-induced and *Ac*MNPV infection-induced caspase activation, we harvested cell lysates of *hhi1*-transfected cells at different time points posttransfection and incubated them in the wells of 96-well plates with immobilized fluorogenic substrates that can be preferentially cleaved by different caspases (ApoAlert caspase assay plates; Clontech).

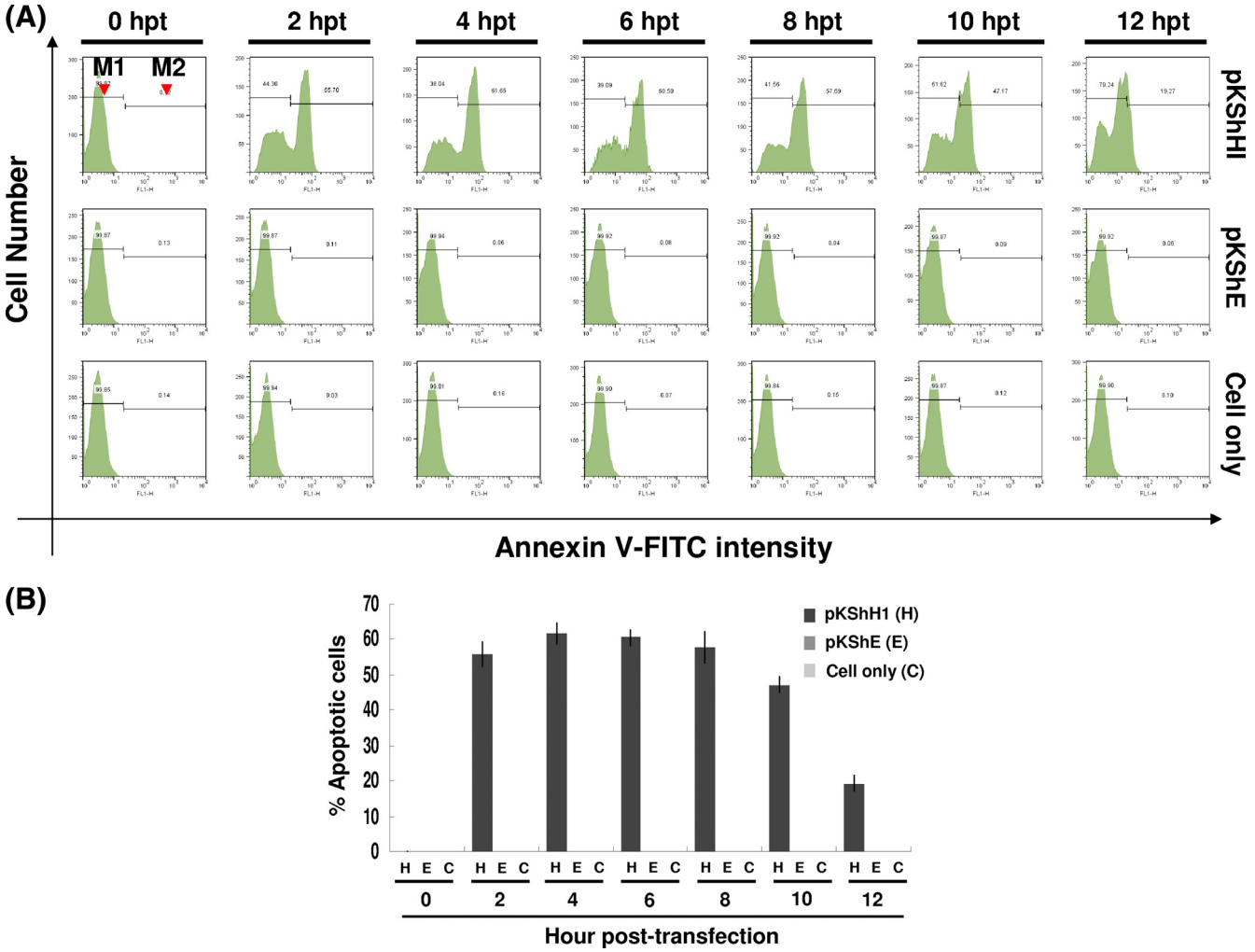

FIG. 2. Assessment of apoptosis resulting from *hhi1* transfection in Sf-21 cells by annexin V assay. (A) Representative histograms of flow cytometry analysis showing the fluorescence intensity of annexin V-labeled apoptotic cells transfected with pKShH1 or pKShE. The annexin V-positive cells can be identified in the M2 region. M1, nonapoptotic cells; M2, apoptotic cells. (B) Bar graph showing the percentage of apoptotic cells at different time points posttransfection resulting from annexin V labeling.

At the same time, untransfected cells were exposed to UV light (50 mJ/cm^2 for 30 min) to induce apoptosis in these cells as a control. The result showed that *hhi1* expression only resulted in the cleavage of DEVD-AMC, an effector caspase substrate (Fig. 4A). Effector caspase activity started to increase at 2 h after *hhi1* transfection and reached a maximum level at 8 h after *hhi1* transfection before declining (Fig. 4A). The decline probably was due to cell death as a result of apoptosis, which subsequently affected the effector caspase activity.

To confirm that *hhi1* can activate caspase, we added the caspase inhibitors Ac-DEVD-CHO (a mammalian caspase-3 inhibitor) and zVAD-fmk (a broad caspase inhibitor) separately to cells transfected with *hhi1* and then detected cell apoptosis by TUNEL assay. DNA fragmentation, the result of apoptosis, was clearly observed in *hhi1*-transfected cells at 16 hpt (Fig. 4B). However, with the addition of the caspase inhibitor Ac-DEVD-CHO or zVAD-fmk, DNA fragmentation was much reduced and appeared only at 24 hpt (Fig. 4B and C). The observation that both Ac-DEVD-CHO and zVAD-fmk could efficiently suppress *hhi1*-induced apoptosis suggests that *hhi1* activates caspase(s) (Fig. 4B and C).

***hhi1* does not activate the processing of pro-*Sf*-caspase-1.** It is well known that the IE1 protein of *Ac*MNPV can induce apoptosis in infected Sf-21 cells. To determine whether IE1 and HHI1 could cause the processing of pro-*Sf*-caspase-1 to its active subunits, we examined lysates of pKShC1-transfected cells (His-tagged pro-*Sf*-caspase-1) (Fig. 5A) with virus infection or additional plasmid transfection. The presence of the His tag allows the detection of full-length pro-*Sf*-caspase-1 and the smaller subunit (P12) of the active enzyme (16, 26). We found that pro-*Sf*-caspase-1 was not processed to its active subunit in mock-transfected and *Hz*NV-1-infected cells (Fig. 5B, lanes 1 and 4). Seshagiri and Miller (44) have shown that both the expression level of pro-*Sf*-caspase-1 and the small subunit (12 kDa) of caspase 1 declined after 24 to 30 hpi upon *Ac*MNPV infection. Similarly, the apoptosis induced by *hhi1* expression may be blocked by the infection of *Hz*NV-1 due to the expression of other viral genes, thereby rendering the pos-

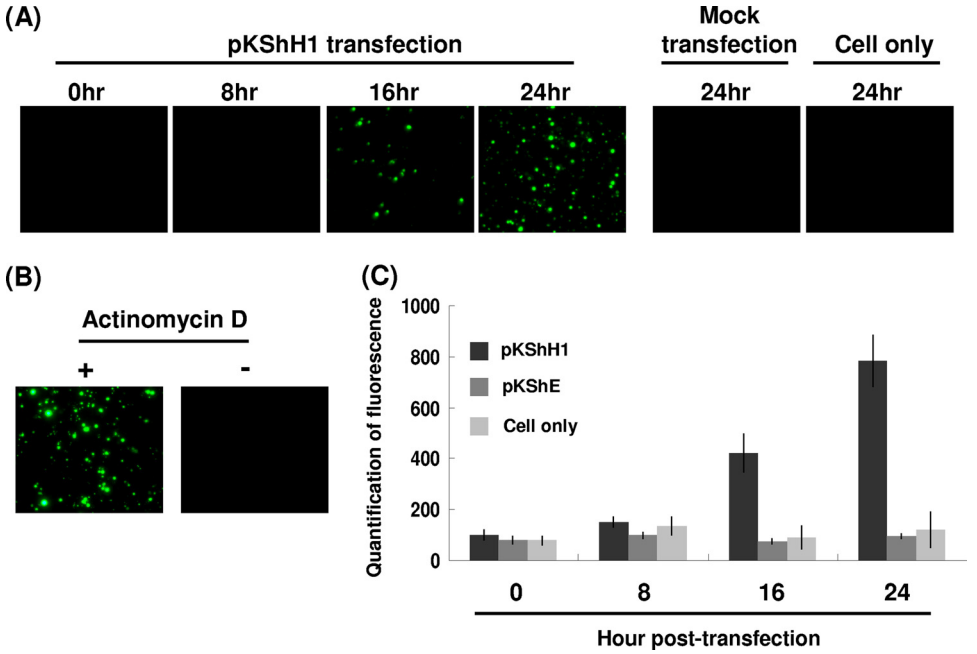

FIG. 3. Assessment of apoptosis resulting from *hhi1* transfection in Sf-21 cells by TUNEL assay. (A) TUNEL assay of Sf-21 cells transfected with pKShH1 at different time points. TUNEL-positive cells were observed in Sf-21 cells transfected with pKShH1 beginning from 16 hpt. (B) Sf-21 cells left untreated or treated with the apoptosis-inducing agent actinomycin D (20 μM) were TUNEL stained at 24 h posttreatment to serve as positive and negative controls, respectively. (C) Quantification of fluorescence of TUNEL-positive cells in Sf-21 cells transfected with pKShH1 or pKShE and Sf-21 cells only at different time points.

sible cleavage of pro-*Sf*-caspase-1 to the small subunit difficult to detect.

To study the function of *hhi1* independently from the effect of other viral genes, *hhi1* (Fig. 5B, lane 5) alone was transfected into cells together with *ie1* alone as a positive control (Fig. 5B, lane 3). In this experiment, the expression level of pro-*Sf*-caspase-1 was detected at 72 hpt, a time point at which the small subunit of caspase-1, which is generated by baculovirus infection, should have been degraded (Fig. 5B, lanes 2) (44). However, without baculovirus interference, we found that the small P12 subunit processed from pro-*Sf*-caspase-1 still was detectable in cells transfected with IE1-expressing plasmid at 72 hpt (Fig. 5B, lane 3). Interestingly, at the same time point, the expression of *hhi1* did not result in the processing of *Sf*-caspase-1 (Fig. 5B, lane 5), suggesting that pro-*Sf*-caspase-1 likely is not processed by *hhi1* transfection.

***Ac-iap2* and *Hz-iap2* inhibit *hhi1*-induced apoptosis.** So far, two groups of baculoviral genes, *p35* and *iap*, are known to suppress cell death (9); however, the genes involved in the antiapoptosis of cells infected with *Hz*NV-1 have not been explored previously. Amino acid sequence analysis showed that two *Hz*NV-1 genes, *orf135* (*Hz-iap1*) and *orf138* (*Hz-iap2*), possess BIR motifs, indicating that they are able to inhibit apoptosis (8). To study whether *hhi1*-induced cell death can be suppressed by the putative antiapoptosis genes mentioned above, we constructed several expression vectors that carry baculovirus *p35*, *Ac-iap1*, *Ac-iap2*, *Hz-iap1*, and *Hz-iap2* gene sequences under the control of the *Drosophila* heat shock 70 promoter (Fig. 6A). The products of all of these genes contain a His tag for the detection of expressed proteins (Fig. 6B). These expression vectors were transfected into Sf-21 cells along with an *hhi1* expression vector. We found that there were fewer cells showing signs of cell death in cells transfected with *Ac-iap2* or *Hz-iap2* under light microscopy (data not shown). Cells then were stained with annexin V, and flow cytometry analysis showed that about 80 to 90% of cells transfected with *p35*, *Ac-iap1*, or *Hz-iap1* became apoptotic (Fig. 6C). In contrast, only 20 to 30% of cells transfected with *Ac-iap2* or *Hz-iap2* showed signs of apoptosis. These results clearly demonstrate that *Ac-iap2* and *Hz-iap2* are capable of suppressing *hhi1*-induced apoptosis (Fig. 6C and D). By using *ie1*-induced apoptosis as a control, we found that *p35* could suppress *ie1*-induced apoptosis (Fig. 6E), which correlated well with previous reports (39, 43). Our results also showed that IAPs of *Ac*MNPV and *Hz*NV-1 had no inhibitory effects on *ie1*-induced apoptosis (Fig. 6E). These results indicate that *hhi1* and *ie1* induce apoptosis in host cells through different pathways, thereby enabling *p35* to suppress *ie1*-induced apoptosis but not *hhi1*-induced apoptosis. In addition, different antiapoptotic genes may act differently on different apoptosis inducers and suppress apoptosis through different mechanisms.

We next performed caspase activity assays and showed that *p35*, *Ac-iap1*, and *Hz-iap1* could not suppress caspase activity induced by *hhi1*. However, *Ac-iap2* and *Hz-iap2* could suppress the activity of caspase by approximately 40% (Fig. 6F). These results showed that *Ac-iap2* and *Hz-iap2* could suppress *hhi1*-induced apoptosis by the suppression of caspase activity. Interestingly, *p35*, the suppressor of apoptosis in insect cells encoded by baculovirus, did not seem to suppress *hhi1*-induced apoptosis.

PAT1, a noncoding RNA, inhibits *hhi1*-induced apoptosis. Previously, we identified the *pag1* gene of *Hz*NV-1, which ex-

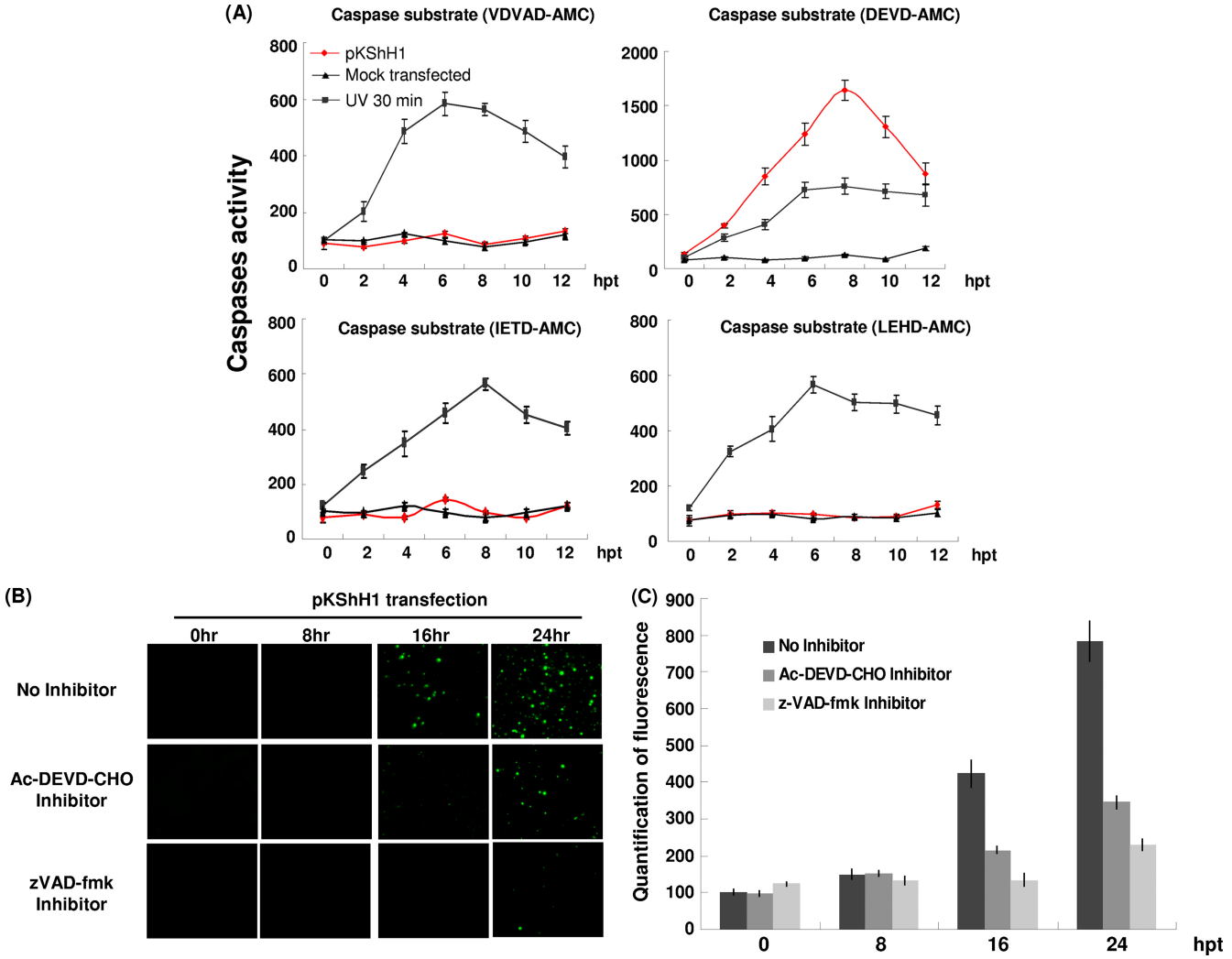

FIG. 4. *hhi1* induces activation of an insect caspase-3-like protein. (A) Assay of the caspase activity induced by *hhi1* transfection. An ApoAlert caspase assay kit (Clontech) was used to measure caspase activities in Sf-21 cells transfected with pKShH1 or pKShE (mock) at 0, 2, 4, 6, 8, 10, and 12 hpt or after being subjected to UV irradiation. The substrates VDVAD-AMC, DEVD-AMC, IETD-AMC, and LEHD-AMC were added to the cell supernatant individually. After incubation for 1 h at 37°C, enzyme activity was measured through fluorogenic cleaved substrates. (B) TUNEL assay of the *hhi1*-transfected cells. Sf-21 cells were transfected with pKShH1 at different time points and then incubated with or without the caspase inhibitor Ac-DEVD-CHO (10 μM) or zVAD-fmk (10 μM) before being subjected to TUNEL assay. TUNEL-positive signals were detected at 16 and 24 hpt in the absence of caspase inhibitor, but signals were clearly detected only at 24 hpt in the presence of the caspase inhibitors. (C) Bar graph representations of the TUNEL assay showing the fluorescence intensity of TUNEL-positive cells at different time points posttransfection with or without caspase inhibitor Ac-DEVD-CHO or zVAD-fmk.

presses a noncoding RNA, PAT1, and is essential for the establishment of latent infection (5, 6). To investigate the possible interaction between *hhi1* and *pag1*, we constructed an expression vector in which *pag1* expression was controlled by its own promoter (51). We transfected *hhi1* into Sf-21 cells with or without *pag1* and observed the transfected cells under a microscope. At 12 hpt, we found prevalent apoptosis in cells transfected with *hhi1* alone, while rather strikingly, significantly fewer apoptotic bodies were observed in cells cotransfected with *hhi1* and *pag1* (Fig. 7A). To estimate the level of apoptosis, transfected cells were stained with annexin V and were analyzed by flow cytometry at 4 hpt. The results showed that the apoptotic cells were reduced from 90% in the *hhi1*-transfected cells to 15% in the cells cotransfected with *hhi1* and *pag1* (Fig. 7B). Previous reports have identified P35 and the proteins belonging to the IAP family as the major antiapoptotic proteins. Our experimental data further identified a noncoding RNA that can serve a similar function in suppressing apoptosis. We currently are trying to elucidate the interaction between *hhi1* and *pag1*, which results in the suppression of *hhi1*-induced apoptosis by *pag1*.

***Hz*NV-1-induced apoptosis can be suppressed by *p35* through activation of *pag1*.** Our previous experiments showed that *p35* could inhibit *Hz*NV-1 superinfection-induced apoptosis in latently infected cells (28). However, our current results show that *p35* could not suppress *hhi1*-induced apoptosis in transient expression assays. We previously noticed that *p35* expression levels varied in different stable cell lines, although

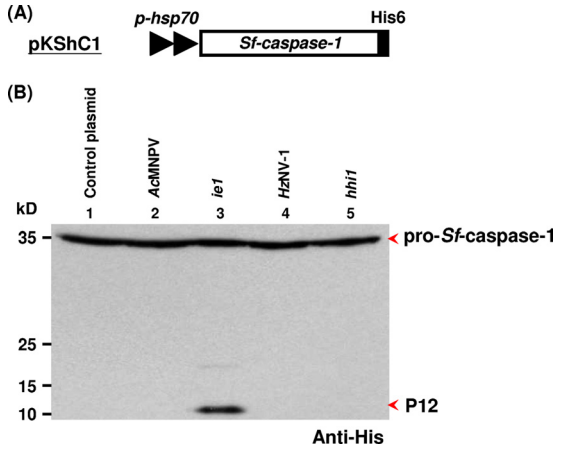

FIG. 5. *hhi1* does not cause the processing of pro-*Sf*-caspase-1. (A) Schematic diagram of plasmid pKShC1, which expresses His-tagged *Sf*-caspase-1. (B) Assay monitoring the processing of *Sf*-caspase-1 by viral infections or plasmid transfections. Sf-21 cells first were transfected with pKShC1. At 4 hpt, cells were inoculated with *Ac*MNPV (MOI, 1; lane 2) or *Hz*NV-l virus (MOI, 1; lane 4). The same His-tagged *Sf*-caspase-1 also was cotransfected into Sf-21 cells with plasmids pKSh (control; lane 1), pKShIEΔH (expresses untagged IE1; lane 3), or pKShHIΔH (expresses untagged HHI1; lane 5) separately. Cells then were collected at 72 hpt, and both the uncleaved pro-*Sf*-caspase-1 and its processed P12 subunit were detected by Western blot analysis using anti-His-tagged antibody (44).

this is quite normal in the stable transfection of a given gene (28). However, what we did not understand was that the levels of PAT1 in the *p35* stably transfected latent cells lines were stimulated to much higher levels than those of parental latent cells (28). Since we have demonstrated that *pag1* can block *hhi1*-induced apoptosis (Fig. 7A and B) and P35 is a well-known transactivator (13, 50), we speculated that the apoptosis inhibition by *p35* reported in Lee and Chao is achieved indirectly through the stimulation of *pag1* expression by *p35* (28). To verify this possibility, we introduced *p35*- and *pag1*-expressing plasmids into cells and detected the expression level of *pag1* in transfected cells by RT-PCR. The result showed that *pag1* expression was stimulated significantly by the expression of *p35*, suggesting that P35 functions as an efficient transactivator for the expression of *pag1* (Fig. 7C). We further confirmed this hypothesis by cotransfecting a *p35* expression plasmid with another plasmid that contains a luciferase reporter gene driven by the *pag1* promoter. The result showed that luciferase activity was increased in the presence of *p35*, indicating that *p35* indeed stimulates the activity of the *pag1* promoter for downstream gene expression (Fig. 7D). Therefore, the blocking of *Hz*NV-1 superinfection-induced apoptosis by *p35* is more likely an indirect result of *pag1* activation, which in turn suppresses *hhi1*-induced apoptosis.

DISCUSSION

Many viral early genes are strong transcriptional activators, and previous experiments have shown that some of them can be strong inducers of apoptosis in infected cells (27, 39, 42). Baculovirus *ie1* was found previously to be capable of inducing apoptosis in Sf-21 cells (25, 39). *icp0* of herpes simplex virus (HSV) and *ns1* of human influenza A virus are two other examples that have been demonstrated to induce apoptosis in host cells (18, 23, 27). Previously, we found that *hhi1*, an early gene and strong transactivator of *Hz*NV-1, can activate viruses in the latently infected cells (5, 6, 51). *pag1*, another early gene, which, interestingly, carries only a noncoding RNA, was shown to function in the establishment and/or enhancement of latent *Hz*NV-1 virus infection in host cells (5). In this study, we found that *hhi1* not only transactivates gene expression but also plays a direct or indirect role in promoting *Hz*NV-1-induced apoptosis. In contrast to apoptosis induced by *ie1* of *Ac*MNPV, a transient expression assay revealed that *hhi1* cannot activate the processing of pro-*Sf*-caspase-1. Therefore, it is likely that *Hz*NV-1 induces apoptosis in host cells through a different caspase pathway. This notion is further supported by the finding that *hhi1*-induced apoptosis can be blocked by *Ac-iap2* and *Hz-iap2* but not *p35*.

Previous reports have shown that apoptosis can be regarded as a cellular defense mechanism against viral infection (9, 28). Viruses, however, also have evolved to produce a group of genes that can suppress or delay cell-induced apoptosis. *p35* and *iap* genes were first discovered in baculovirus *Ac*MNPV (12). Although *p35* has been shown to block the apoptosis triggered by *ie1* during baculovirus infection, since their discovery the exact functions of the *Ac-iap* genes have not been determined (10). Since viruses should retain only genes that they need, it is interesting to speculate on the possible roles of these *iap* genes (9). In this paper, we found that *iap2* of both *Hz*NV-1 (8) and *Ac*MNPV (3, 15) can effectively suppress apoptosis induced by *hhi1*.

P35 originally was identified as a general caspase inhibitor due to its ability to inhibit virus-induced caspase activity (44). Many reports have demonstrated that *p35* can suppress apoptosis induced by various genes in Sf-21 cells (9, 16, 26); however, our result shows that *p35* cannot suppress *hhi1*-induced apoptosis in this cell line. Thus, our data suggest that *hhi1* induces apoptosis by activating a caspase other than *Sf*-caspase-1. It has been demonstrated that *p35* fails to rescue cell death induced by a caspase called DRONC, *in vivo* and *in vitro*, in *Drosophila* cells (19, 36). In addition, a novel caspase distinct from *Sf*-caspase-1 that is not sensitive to *p35* also was proposed to exist in Sf-9 cells (1). All of these are indications that caspases other than *Sf*-caspase-1 exist in insect cells, and one of them may be responsible for the *hhi1* induction of apoptosis.

The main effector caspase in Sf-21 cells is *Sf*-caspase-1 (32). *Sf*-caspase-1 is generated from pro-*Sf*-caspase-1 (P37), which can be cleaved into short prodomains P6, P12, and P19 by a sequential proteolytic process upon apoptotic signaling (1, 16, 26, 30). In our experiment, we showed that *ie1*, but not *hhi1*, can activate *Sf*-caspase-1 cleavage (43). Out of four mammalian caspase substrates, we found that *hhi1* induces the cleavage of DEVD-AMC only (caspase 3 substrate). However, previous reports showed that mammalian caspases 6, 7, 8, and 10, as well as *Sf*-caspase-1, all can act on substrate DEVD-AMC (16, 26, 37). This raises the possibility that multiple caspases are present in Sf-21 cells, and one of them may be activated by *hhi1* to cleave the substrate DEVD-AMC. Further studies are required to see whether this unique caspase(s) can be found in Sf-21 cells. Although it seems unlikely, *hhi1* may also act on

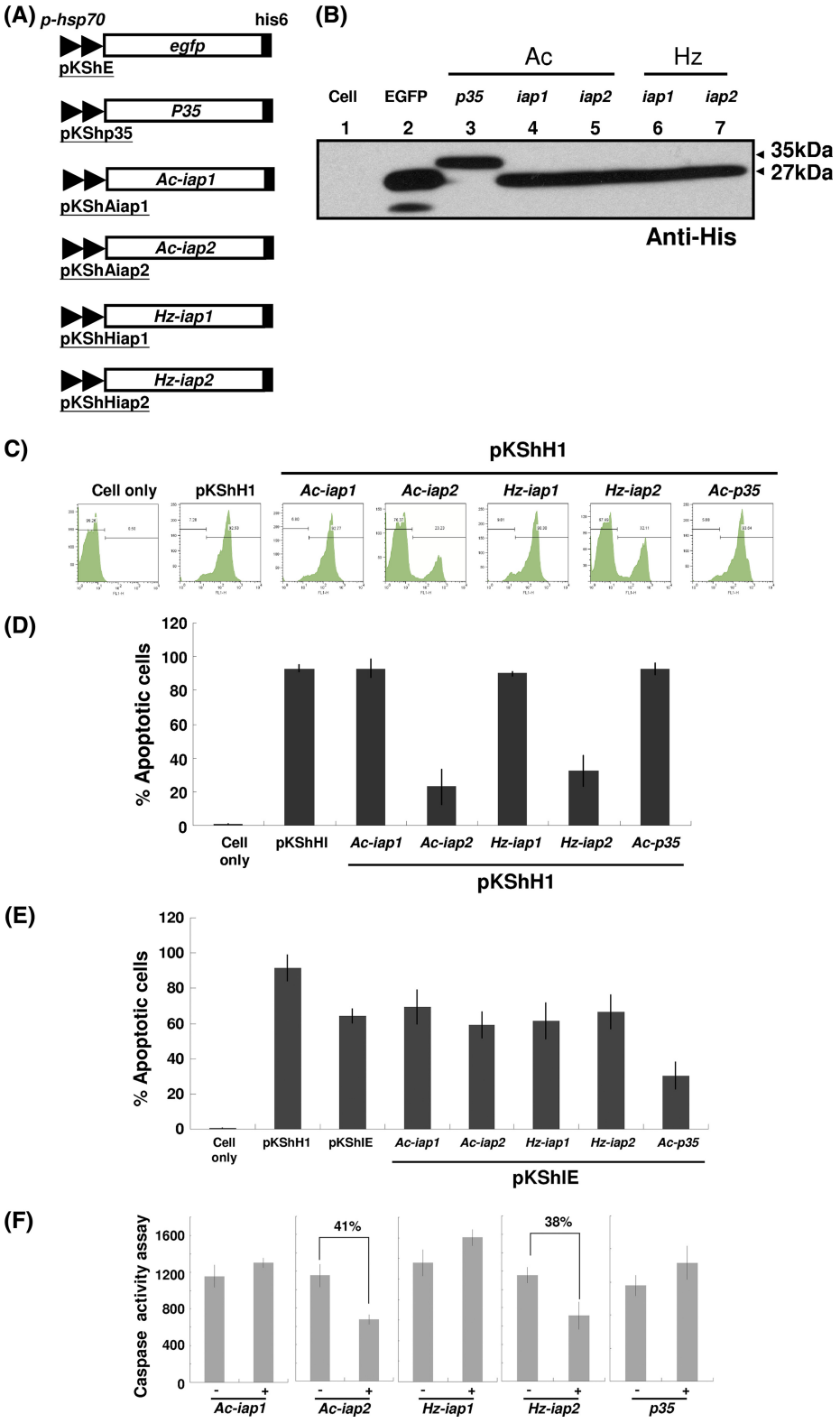

FIG. 6. Effects of antiapoptosis genes on *hhi1*-induced apoptosis in Sf-21 cells. (A) Schematics of plasmids containing antiapoptosis genes. (B) Western blot analysis showing proper transient expression of antiapoptotic proteins from the plasmids shown in panel A. (C) Annexin V assay of apoptosis induction in Sf-21 cells by flow cytometry. Sf-21 cells were transfected with either *hhi1* (pKShH1) alone or with anti-apoptosis gene *Ac-iap1*, *Ac-iap2*, *Hz-iap1*, *Hz-iap2*, or *p35* (0.5 μg of each plasmid). Transfected cells were stained by annexin V at 2 hpt and then analyzed by flow cytometry. Apoptotic cells that stained positive for annexin V were identified in the M2 region. (D) Bar graph representation of the percentages of apoptotic cells using the data shown in panel C. (E) Apoptosis induction assay in Sf-21 cells transfected with *hhi1*, *ie1*, or *hhi1* and antiapoptosis genes. (F) Caspase-specific activity assay in *hhi1*-transfected Sf-21 cells, which were cotransfected with or without anti-apoptosis genes, using DEVD-AMC as a substrate. Percentages of apoptosis suppression by *Ac-iap2* and *Hz-iap2* are indicated.

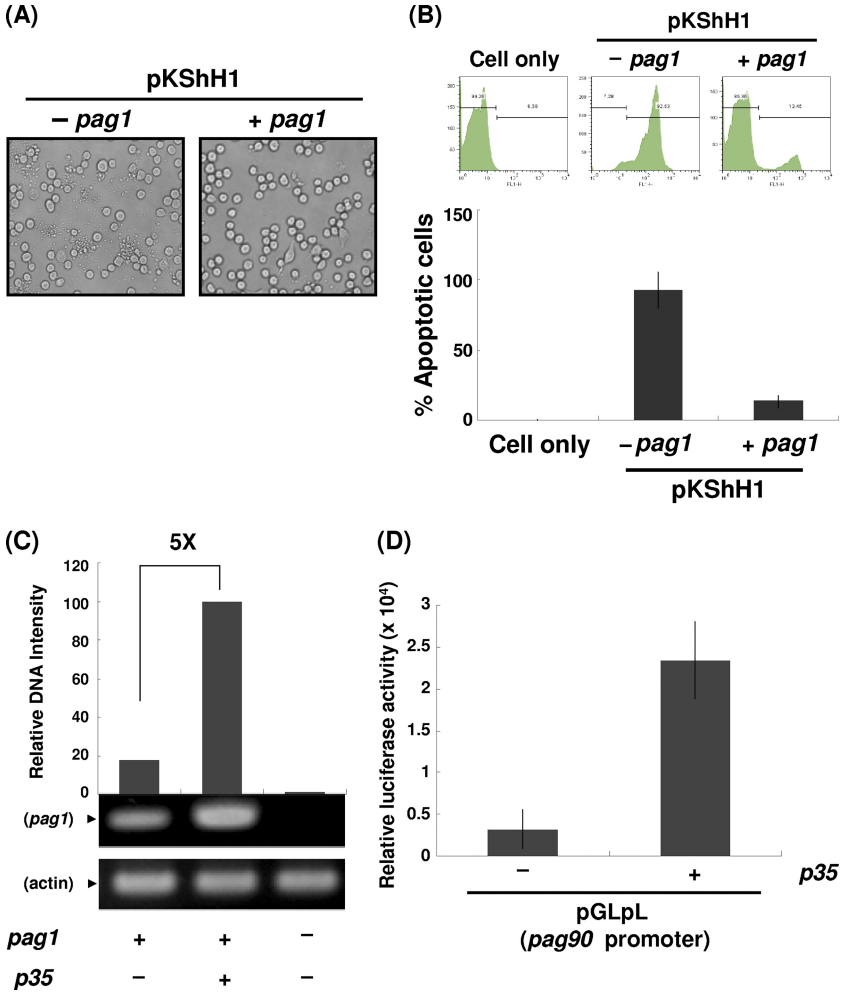

FIG. 7. Analysis of antiapoptotic activities of *pag1* in Sf-21 cells. (A) Inhibition of *hhi1*-induced apoptosis by *pag1*. Sf-21 cells (2×10^5 cells per 24-well plate) were transfected with pKShH1 in the presence or absence of pKSpP1 (carrying *pag1*). At 12 hpt, cells were examined by light microscopy and photographed to examine the inhibition of apoptosis. (B) Flow cytometry analysis of apoptosis suppression. In this assay, apoptotic cells that stained positive by annexin V were identified in the M2 region. The bar graph below shows the percentages of apoptotic cells derived from the flow analysis. (C) PAT1 level analysis by RT-PCR. Sf-21 cells were transfected with plasmids pKSpP1 (*pag1*) alone or cotransfected with pKShP35 (carrying *p35*), and the expression of the *pag1* gene product, PAT1, was analyzed by RT-PCR. The expression of actin served as a control. (D) Luciferase assay showing that *p35* can activate the *pag1* promoter. Plasmid pGLpL (*luciferase* coding region driven by the *pag1* promoter) (51) was cotransfected with or without pKShP35 into Sf-21 cells. Luciferase activities from each group were measured at 48 hpt.

genes downstream of *Sf*-caspase-1 and simply bypass *Sf*-caspase-1 in the caspase-induced apoptosis pathway.

Besides *Sf*-caspase-1, the effector caspase *Sf*-caspase-2 was identified previously (52). During baculovirus infection, the proteolytic processing of effector caspases *Sf*-caspase-1 and *Sf*-caspase-2 can be prevented by the caspase inhibitor P49. Although P49 is a homolog of pancaspase inhibitor P35, the latter prevents the proteolytic processing of *Sf*-caspase-1 but not *Sf*-caspase-2 (16, 26). Therefore, we cannot rule out the possibility that *hhi1* induces apoptosis by activating *Sf*-caspase-2.

In Fig. 5B, although we used *ie1* as a control, the small subunit P12 of pro-*Sf*-caspase-1 still was clearly detectable at 72 hpt in the transfected cells (Fig. 5B, lane 3); we could not see the same subunit resulting from *hhi1* transfection. Previously, Seshagiri and Miller (44) showed that P12 could result from the cleavage of pro-*Sf*-caspase-1 (driven by a heat shock promoter). The P12 fragment was detected at around 12 hpi and degraded afterwards. In our experiments shown in Fig. 5, although heat shock promoters were used to drive *ie1* (lane 3) and *hhi1* (lane 5), cells were not heat shock treated or virus infected as in Seshagiri and Miller (44). Under these conditions, we could clearly see the existence of P12 until 72 h after *ie1* transfection, suggesting that this cleaved subunit can stay in the cell for long periods without virus infection. To further validate these results, we performed a Western blot experiment to measure caspase-1 expression levels at different time points (6 to 72 hpt) and found that the level of pro-*Sf*-caspase-1 was highest at 72 hpt (see Fig. S1 in the supplemental material). Further experiments showed that P12 cleaved from the cotransfection of pro-*Sf*-caspase-1 and *ie1* began to be detectable at 48 hpt, and the expression level increased up to 72 hpt (see Fig. S2, lanes 3, in the supplemental material). This is an indication that P12 is not further degraded without the infec-

tion of the virus. Nevertheless, under the same conditions, we still could not detect P12 as a result of *hhi1* transfection (see Fig. S2).

There are several points worth mentioning here. First, with *Ac*MNPV coinfection, P12 was not detected at all (Fig. 5, lanes 2; also see Fig. S2). It is likely that the cleaved P12 was quickly degraded, as shown in Seshagiri and Miller (44). It also is likely that as the MOI we used was 1, rather than 20 as used by Seshagiri and Miller (44), the resulting subunit was present at levels too low to be detected in these experiments. However, P12 was detectable by transfection with *ie1*, suggesting that transfection was successful in our studies. Second, although P12 was not detected after *hhi1* transfection (Fig. 5, lanes 5; also see Fig. S2), *hhi1* transfection clearly resulted in strong apoptosis (date not shown), suggesting that the *hhi1* plasmid used in this experiment was functional.

In the current study, we found that *p35* fails to suppress *hhi1*-induced apoptosis; however, our previous report showed that *p35* can suppress apoptosis induced by *Hz*NV-1 superinfection in latently infected cells (28). This apparent contradiction can be explained by viewing *p35* in a different role. Previously, we have shown that *p35* can activate early *Hz*NV-1 genes by functioning as a transactivator (50). Here, we further showed that *p35* can function as a strong transactivator of the *pag1* promoter (Fig. 7C and D), and our new discovery found that *pag1* is a strong inhibitor of *hhi1*-induced apoptosis (Fig. 7A and B). Since the *pag1* product PAT1 is a noncoding RNA (5), the mechanism by which *pag1* inhibits apoptosis may be different from those exploited by the known antiapoptosis proteins P35 and the IAPs.

One possible mechanism by which PAT1, the product of *pag1*, could inhibit apoptosis is by the production of microRNAs (miRNAs) (46). Similarly to PAT1, HSV also expresses a noncoding RNA, the latency-associated transcript (LAT), which has been shown to inhibit virus-induced apoptosis (23, 45). These studies showed that miRNAs are produced from the LAT to block the translation of the early genes *icp0* and *icp4* (46). Although further experiments are needed, it is reasonable to assume that virus will go into latency if the expression of major early transcripts is blocked. We are currently investigating how *pag1* suppresses apoptotic cell death, and the results should help us to understand the roles played by noncoding RNAs in insects.

In this study, we have employed a transient assay to study the utilization of *Hz-iap2* and *pag1* by *Hz*NV-1 virus to suppress apoptosis. *Hz*NV-1 virus expresses both *pag1* and *Hz-iap2* during productive viral infection but only expresses *pag1* during viral latency (5). Hence, the roles of *Hz-iap2* and *pag1* may overlap but functionally differ. It is likely that *Hz*NV1 virus mainly uses *Hz-iap2* to suppress apoptosis triggered by *hhi1* expression during initial viral invasion into healthy cells. In other words, *Hz-iap2* only functions and is used during productive viral infection. *pag1*, on the other hand, may be used during productive infection to control the excess *hhi1* transcript and may serve as the major force to suppress possible viral reactivation initiated by *hhi1* expression (51) during latent viral infection. *pag1* also may be responsible for blocking possible apoptosis induced by reactivated *hhi1* during viral latency.

In conclusion, we have shown that the early gene *hhi1* of *Hz*NV-1 may promote apoptosis directly or indirectly in transiently transfected Sf-21 cells. It has been reported that some virus-encoded transcription factors, such as baculovirus IE1 and human immunodeficiency virus Tat, are proapoptotic, whereas some DNA viruses trigger apoptosis via the expression of transcription factors involved in viral DNA replication or perturbing the normal cell cycle (2, 43). Thus, *hhi1* might induce apoptosis by either activating the expression of prodeath genes or altering the cell cycle of the host. The apoptosis pathway triggered by HHI1 does not seem to result in the activation of *Sf*-caspase-1 (Fig. 5B) but does result in the cleavage of mammalian caspase 3 substrate. We also identified a functional antiapoptosis gene, *Hz-iap2*, which can suppress *hhi1*-induced apoptosis. Interestingly, although *Ac-iap2* was speculated to be a nonfunctional *iap* (9), we found that it is able to block apoptosis induced by *hhi1*. Furthermore, although *p35* cannot directly suppress *hhi1*-induced cell death, we have shown an indirect mechanism whereby *p35* can stimulate the expression of the *pag1* gene to inhibit apoptosis. We conclude that *hhi1* provides a unique opportunity for the further detailed investigation of apoptosis induction and suppression through both caspase-dependent and -independent pathways.

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