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Baculovirus DNA Replication-Specific Expression Factors Trigger Apoptosis and Shutoff of Host Protein Synthesis during Infection[▽]

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Apoptosis is an important antivirus defense. To define the poorly understood pathways by which invertebrates respond to viruses by inducing apoptosis, we have identified replication events that trigger apoptosis in baculovirus-infected cells. We used RNA silencing to ablate factors required for multiplication of *Autographa californica* multicapsid nucleopolyhedrovirus (Ac*M*NPV). Transfection with double-stranded RNA (dsRNA) complementary to the Ac*M*NPV late expression factors (*lef*s) that are designated as replicative *lef*s (*lef-1*, *lef-2*, *lef-3*, *lef-11*, *p143*, *dnapol*, and *ie-1*/*ie-0*) blocked virus DNA synthesis and late gene expression in permissive *Spodoptera frugiperda* cells. dsRNAs specific to designated nonreplicative *lef*s (*lef-8*, *lef-9*, *p47*, and *pp31*) blocked late gene expression without affecting virus DNA replication. Thus, both classes of *lef*s functioned during infection as defined. Silencing the replicative *lef*s prevented Ac*M*NPV-induced apoptosis of *Spodoptera* cells, whereas silencing the nonreplicative *lef*s did not. Thus, the activity of replicative *lef*s or virus DNA replication is sufficient to trigger apoptosis. Confirming this conclusion, Ac*M*NPV-induced apoptosis was suppressed by silencing the replicative *lef*s in cells from a divergent species, *Drosophila melanogaster*. Silencing replicative but not nonreplicative *lef*s also abrogated Ac*M*NPV-induced shutdown of host protein synthesis, suggesting that virus DNA replication triggers inhibition of host biosynthetic processes and that apoptosis and translational arrest are linked. Our findings suggest that baculovirus DNA replication triggers a host cell response similar to the DNA damage response in vertebrates, which causes translational arrest and apoptosis. Pathways for detecting virus invasion and triggering apoptosis may therefore be conserved between insects and mammals.

DNA and RNA viruses are potent inducers of apoptosis. This host suicide response is an effective antiviral strategy because the destruction of virus-infected cells can significantly diminish virus yields and affect pathogenicity. Supporting the significance of apoptosis as an antiviral defense in invertebrates, insect viruses carry diverse genes that suppress apoptosis and facilitate virus multiplication (reviewed in references 5, 6, and 14). Importantly, the apoptotic response may also influence the competency of insects, including mosquitoes, to function as vectors of human viral pathogens (16, 56). Thus, a better understanding of the molecular mechanisms by which viruses trigger apoptosis in invertebrates and how pathogenesis is affected will provide a critical framework for control of insect-vectored diseases.

The baculoviruses provide an attractive model for defining the mechanisms by which insect viruses trigger apoptosis and thus influence virus dissemination and pathogenesis within an animal. These large, double-stranded DNA viruses cause widespread apoptosis during infection of insects, which include the larvae of moths and butterflies (order Lepidoptera) (5, 6, 14, 49). The baculoviruses encode potent antiapoptotic proteins that counter the host cell response by suppressing premature cell death and thus promoting prolific multiplication. In particular, baculovirus inhibitor-of-apoptosis (IAP), P35, and P49 inhibit the activation or activity of the death proteases known as caspases and thus prevent apoptosis, even in diverse organisms, including mammals (6, 14, 39). In the absence of virus-encoded apoptotic suppressors, baculovirus production and spread within infected larvae is severely restricted (4). Thus, in permissive insects, apoptosis serves to limit virus reproduction and pathology and can be considered an effective innate immune response (5, 7).

The best-studied model for virus-induced apoptosis in invertebrates is the prototype baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus (Ac*M*NPV). In the absence of antiapoptotic genes, this 134-kb genome DNA virus induces widespread caspase-dependent apoptosis of permissive *Spodoptera frugiperda* (SF21) cells. This host response severely limits Ac*M*NPV yields and destroys the infected cell by a rapid process that involves chromatin condensation, DNA fragmentation, plasma membrane blebbing, and cytolysis (8, 9, 19, 33). Although virus interaction with host cell receptors is insufficient to trigger apoptosis (25), the virus genes or replication events directly responsible for apoptosis are unknown.

Inhibition of Ac*M*NPV DNA synthesis either by using the DNA polymerase inhibitor aphidicolin or temperature-sensitive Ac*M*NPV mutations reduces virus-induced apoptosis (10, 25). In addition, the activation of host cell caspases coincides with the initiation of viral DNA synthesis (25, 26). These findings suggested that the apoptotic signal is linked to virus DNA replication. However, it was unclear whether DNA replication is the direct trigger or if virus DNA synthesis is indirectly involved because it promotes late multiplicative events, which could trigger apoptosis. The Ac*M*NPV immediate-early protein IE1 is also necessary for virus-induced apoptosis (46). Because this transcriptional activator is required for viral DNA

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replication and replication-dependent late gene expression (43, 46, 48), it may trigger apoptosis by initiating DNA synthesis or promoting downstream multiplicative events. Alternatively, IE1 may directly activate host pro-death genes, perturb the cell cycle, or inhibit host biosynthetic pathways, all of which are sufficient to cause apoptosis (reviewed in references 18, 24, 29, 30, and 57).

To distinguish the replication events responsible for baculovirus-induced apoptosis, we used RNA silencing to ablate the factors required for Ac*M*NPV multiplication in permissive, apoptosis-sensitive *Spodoptera* cells. RNA silencing is an effective means by which to selectively reduce viral or cellular gene expression during infection of invertebrates (13, 35, 46, 51). An important advantage to our approach was the capacity to evaluate the function of individual genes during replication of fully infectious virus after normal receptor-mediated entry. Thus, RNA silencing can be more informative than other approaches, such as the overexpression of specific viral genes in the absence of other contributing genes. We used here RNA silencing as a unique means to distinguish the proapoptotic contributions of virus DNA replication from those of viral late gene expression. By selectively silencing components of the Ac*M*NPV-encoded RNA polymerase that is responsible for late and very late virus transcription (reviewed in references 14 and 41), the effects of DNA replication on the host cell were disengaged from those involving late virus gene expression.

We report here that silencing genes essential for Ac*M*NPV DNA replication, defined as replicative late expression factors (*lef*s), suppressed Ac*M*NPV-induced apoptosis. In contrast, silencing genes that are selectively required for virus late gene expression, defined as nonreplicative *lef*s, had no effect on virus-induced apoptosis. Thus, the activities of the Ac*M*NPV replicative *lef*s or events associated with virus DNA replication provide the critical apoptotic signal in the infected cell, including those of the distantly related species *Drosophila melanogaster*. The replicative but not the nonreplicative *lef*s also contributed to the inhibition host protein synthesis, which is characteristic of Ac*M*NPV infection. Our study therefore suggests that analogous to certain DNA viruses of vertebrates, the baculoviruses trigger a DNA damage and translational arrest response that culminates in apoptosis of the host cell.

MATERIALS AND METHODS

Cells. *Spodoptera frugiperda* IPLB-SF21 cells (55) were propagated in TC100 growth medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories). *Drosophila melanogaster* Schneider DL-1 cells (44) were propagated in Schneider's growth medium (Invitrogen) supplemented with 15% heat-inactivated FBS.

Viruses. Wild-type (wt) L-1 strain Ac*M*NPV (28) and Ac*M*NPV recombinants wt/lacZ (*p35*$^+$ *polh*$^-$ *lacZ*$^+$), vΔ35K/lacZ (*p35*$^-$ *polh*$^-$ *lacZ*$^+$), vP35 (*p35*$^-$ *polh*$^-$ *ie-1*^{prm}-*p35*$^+$ *lacZ*$^+$), and vOpIAP (*p35*$^-$ *polh*$^-$ *ie-1*^{prm}-*Op-iap*$^+$ *lacZ*$^+$) were described previously (19, 27, 60). The very late promoter of the *polyhedrin* gene (*polh*) directs expression of *lacZ* of recombinant wt/lacZ. The promoter of the immediate-early gene *ie-1* directs expression of *p35* and *Opiap3* from the *polh* locus of recombinants vP35 and vOpIAP, respectively. For inoculation, extracellular budded virus in TC100 plus 10% FBS was added to SF21 and DL-1 monolayers using 10 and 20 PFU/cell, respectively. After rocking the samples for 1 h at room temperature, the inoculum was replaced with FBS-supplemented growth medium, and the cells were incubated at 27°C.

Plasmids. pBluescript K/S$^+$ (Invitrogen) plasmids containing portions of the enhanced green fluorescence protein gene (*egfp*) or the Ac*M*NPV genes *gp64* or *ie-1* used as templates for in vitro transcription reactions were described previously (46). To generate plasmids with Ac*M*NPV genes *p143* and *lef-3*, a 1,200-bp SacI fragment from plasmid pRESTA-Acp143ORF and a 1,575-bp XbaI-XhoI fragment from pRESTB-Aclef3 (kindly provided by Eric Carstens, Queen's University), respectively, were inserted into pBluescript K/S$^+$. All other baculovirus genes or fragments thereof were cloned by PCR amplification of the Ac*M*NPV genome (GenBank accession number NC_001623). The full-length open reading frames (ORFs) of *lef-1*, *lef-2*, *lef-11*, *p47*, and *lef-9* or a fragment of the ORF of *dnapol* (ORF nucleotides 61 to 862), *lef-8* (ORF nucleotides 427 to 1,838), and *pp31* (ORF nucleotides 94 to 837) were each inserted into pBluescript K/S$^+$. The genomic sequences used to generate *lef*-specific double-stranded RNA (dsRNA) were selected so as not to overlap the ORFs of other Ac*M*NPV *lef*s with two exceptions. Our *ie-1*/*ie-0*-specific dsRNA silenced both *ie-1* and *ie-0* due to the overlapping nature of the spliced *ie-0* and unspliced *ie-1* mRNAs (46). The *lef-11*-specific dsRNA overlapped the *pp31* RNA transcript by 136 nucleotides and was expected to ablate *pp31* expression; synthesis of PP31 during infection was reduced in *lef-11* dsRNA-transfected cells (data not shown). Our *pp31*-specific dsRNA did not overlap *lef-11*.

dsRNA transfections. Single-stranded RNA was synthesized by using in vitro transcription reactions (Ampliscribe T3 and T7 kits; Epicentre) with linearized pBluescript K/S$^+$ plasmids as a template. Complementary RNAs were heated to 65°C and cooled 1°C per min to generate dsRNA. SF21 and DL-1 cells were transfected with a dsRNA-liposome mix as described previously (46). Visual inspection revealed that the dsRNAs tested here had no obvious effect on cell viability.

Immunoblots. Infected or mock-infected cells were collected by centrifugation, lysed in 1% sodium dodecyl sulfate (SDS)–1% β-mercaptoethanol, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). After protein transfer, the nitrocellulose membranes were incubated with the following antisera diluted as indicated in parentheses: polyclonal anti-IE1 (1:10,000) (40), monoclonal AcV_5 anti-GP64 (a gift from Gary Blissard, Cornell University) (1:500) (20), and mouse monoclonal anti-actin (1:5,000 dilution) (BD Biosciences). Signal development was conducted as described previously (46). Films were scanned at 300 dpi by using an Epson TWAIN Pro scanner and prepared by using Adobe Photoshop CS2 and Adobe Illustrator CS2.

β-Galactosidase assays. SF21 monolayers were infected with Ac*M*NPV wt/lacZ (multiplicity of infection [MOI] = 10) 24 h after dsRNA transfection. The cells were collected 48 h later, washed, and lysed in Tropix buffer (Galacto-Light Plus kit; Applied Biosystems). The β-galactosidase activity was measured according to the manufacturer's instructions and is reported as the average activity ± the standard deviation determined from triplicate infections.

Cell survival assays. Intact, nonapoptotic SF21 or DL-1 cells were counted after inoculation with Ac*M*NPV recombinant vΔ35K or vOpIAP, respectively, by using a phase-contrast microscope (Axiovert 135TV; Zeiss) and IP Lab Spectrum P software (BD Biosciences) as described previously (21). The mean ± the standard deviation of surviving cells was determined from six nonoverlapping fields of view from triplicate infections and normalized to that of uninfected cells transfected with control *egfp* dsRNA.

Quantitation of Ac*M*NPV DNA. At the indicated times after infection, cells were collected, washed, and suspended in 10 mM Tris (pH 8.0)–1 mM EDTA. SDS and proteinase K were added to 0.2% and 0.1 mg per ml, respectively. After 4 h at 37°C, the mixture was phenol-chloroform extracted and treated with 120 μg of RNase A/ml. Nucleic acid was precipitated with ethanol and suspended in 10 mM Tris (pH 8.0)–1 mM EDTA. Quantitative real-time PCR was performed using 50-μl reactions containing DNA extracted from the equivalent of 25 SF21 cells or 50 DL-1 cells in 1× *Taq* buffer A (Promega), 5.5 mM $MgCl_2$, 90 nM SuperROX reference dye (Biosearch Technologies), 200 nM deoxynucleoside triphosphates, 100 nM DNA probe, and 1 U of *Taq* polymerase (Promega). Because the content of *Spodoptera frugiperda* cellular DNA does not increase during Ac*M*NPV infection (1), nucleic acid samples were normalized for cell equivalents by using the *sfiap* gene from *Spodoptera* (22) (provided by John Reed, Burnham Institute) as a genome standard for SF21 cells. Likewise, the β-*actin* gene from *Drosophila* (15) (provided by Paul Ahlquist, University of Wisconsin-Madison) was used as a DL-1 genome standard. Ac*M*NPV DNA from purified extracellular budded virus and linearized plasmids containing *sfiap* or β-*actin* were used to generate the standard curves used for quantitation. The PCR cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 56°C for 1 min on a real-time PCR machine (7900HT; Applied Biosystems). The following oligonucleotides were used for primers and probes: *sfiap*, forward primer 5′-ATTGGAAGAACCATGACGTACCCT-3′ (50 nM), reverse primer 5′-ACGTATTCTCGACCCTTCACCAAT-3′ (300 nM), and probe 5′-TET-ACAACACGCAAGGTGGTTTGACCGTTGC-TAMRA-3′; *Drosophila* β-*actin*, forward primer 5′-TGACCGACTACCTGATGAAGAT CC-3′ (300 nM), reverse primer 5′-GCAACATAGCACAGCTTCTCCTTG-3′ (300 nM), and probe 5′-FAM-TTTCACCACCACCGCTGAGCGTGAAAT-

TABLE 1. Requirement for Ac*M*NPV replicative and nonreplicative *lef*s

Virus gene	Requirement for[a]: DNA replication	Late gene expression	Virus apoptosis	Gene function/homology[b]
ie-1/ie-0	+	+	+	Transcription activator, binds DNA origins
p143	+	+	+	Helicase, binds DNA, ATPase
lef-1	+	+	−	DNA primase activity, LEF2 association
lef-2	+	+	+	LEF1 primase accessory factor
lef-3	+	+	+	Single-strand DNA binding, P143 association
dnapol	+	+	+	DNA polymerase, 3′→5′ exonuclease
lef-11	+	+	+/−	Unknown function
p47	−	+	−	Late RNA polymerase subunit
lef-8	−	+	−	Late RNA polymerase subunit
lef-9	−	+	−	Late RNA polymerase subunit
pp31	−	+	−	Binds DNA, virogenic stroma association

[a] The requirement of each Ac*M*NPV gene for virus DNA replication (Fig. 3), late gene expression (Fig. 1), and host cell apoptosis (Fig. 5) upon infection of *Spodoptera frugiperda* SF21 cells is denoted by a "+" (required) or a "−" (not required).

[b] The function and properties of the Ac*M*NPV replicative and nonreplicative *lef*s have been reviewed recently (14, 41, 52).

TAMRA-3′; and Ac*M*NPV HindIII-T genome fragment, forward primer 5′-AT TTAACATCGGGCGTGTTAGCTT-3′ (900 nM), reverse primer 5′-TCGCTT TCTAACGTGTTGTCTGAAT-3′ (900 nM), and probe 5′-FAM-CGATTTTG CCATAGCCACACGACGCCT-TAMRA-3′.

Protein radiolabeling. At the indicated times after infection, the growth medium above SF21 monolayers was replaced with phosphate-buffered saline (pH 6.2) (28) containing 200 μCi of Trans^{35}S-label (1,175 Ci/mmol, methionine at 70%; cysteine at ≤15%; MP Biomedical, LLC)/ml. After 1 h at 27°C, the cells were dislodged, collected by centrifugation, and lysed with 1% SDS–1% β-mercaptoethanol. The lysates were subjected to SDS-PAGE and autoradiography.

RESULTS

RNA silencing of Ac*M*NPV *lef*s prevents very late gene expression. To define the virus genes and replication events involved in triggering baculovirus-induced apoptosis, we used dsRNA-mediated silencing to selectively knock down the expression of Ac*M*NPV genes required for essential replicative processes. A principal advantage to this approach is the capacity to evaluate gene function during replication initiated by normal receptor-mediated entry of fully infectious virus (46). By generating gene-specific dsRNA, which was used to transfect cultured cells 24 h prior to inoculation, we ablated viral proteins necessary for Ac*M*NPV DNA replication and late gene expression. To this end, we generated dsRNAs complementary to Ac*M*NPV *ie-1*/*ie-0*, *p143*, *lef-1*, *lef-2*, *lef-3*, *lef-11*, and *dnapol*, which are designated replicative *lef*s because each gene is required for DNA replication and late gene expression in transient-transfection assays (Table 1). Similarly, we synthesized dsRNAs complementary to *p47*, *lef-8*, *lef-9*, and *pp31*, which are designated as nonreplicative *lef*s because each is required for late gene expression but not virus DNA replication (Table 1). In particular, we chose *p47*, *lef-8*, and *lef-9* because they are components of the Ac*M*NPV RNA polymerase complex responsible for late viral transcription (14, 41). As such, ablation of the late RNA polymerase was expected to suppress late and very late multiplicative events.

We first showed that dsRNA had no deleterious effects. Transfection with control dsRNA, including *egfp*-specific dsRNA, did not affect the viability or morphology of mock-infected SF21 cells (Fig. 1Ai). Moreover, upon infection with wild-type Ac*M*NPV, occluded virus particles accumulated in ≥95% of *egfp* dsRNA-transfected cells, as expected (Fig. 1Aii). The synthesis of essential Ac*M*NPV proteins, including envelope fusion protein GP64 and transactivators IE1 and IE0, was comparable to that in untreated cells (Fig. 1B, compare lanes 2 to 6 and lanes 8 to 12). Thus, control dsRNA had a minimal effect on synthesis of Ac*M*NPV proteins or replication events. In contrast, *gp64*-specific dsRNA ablated GP64 synthesis without affecting transactivators IE1 or IE0 (Fig. 1B, lanes 14 to 18). Likewise, *ie-1*/*ie-0* dsRNA, which is complementary to the overlapping *lef*s *ie-1* and *ie-0* (46), ablated these early proteins without affecting GP64 (Fig. 1B, lanes 20 to 24). Thus, gene-specific dsRNA was effective in knockdown of Ac*M*NPV gene products during infection, as shown previously (46).

Upon transfection of SF21 cells, dsRNA specific for each of the replicative *lef*s reduced accumulation of occluded virus produced by wild-type Ac*M*NPV. Occluded virus levels were comparable to that effected by *p143*-specific dsRNA (Fig. 1Aiii and data not shown), in which virus was evident in only 5 to 10% of the cells and in lower quantities on a per cell basis. Likewise, occluded virus was reduced to comparably low levels by dsRNAs specific to the nonreplicative *lef*s, including that for *p47* (Fig. 1Aiv). To quantify the inhibitory effect of *lef* silencing on very late gene expression, we monitored β-galactosidase production in dsRNA-treated cells infected with Ac*M*NPV recombinant wt/lacZ, in which the very late *polh* promoter directs expression of a *lacZ* reporter. Very late expression of *lacZ* in cells transfected with dsRNA specific to replicative *lef*s *ie-1*/*ie-0*, *p143*, *lef-1*, *lef-2*, *lef-3*, *lef-11*, or *dnapol* was reduced from 4- to 20-fold compared to that of cells transfected with control *egfp* dsRNA (Fig. 1C). Transfection with dsRNA specific to each of the nonreplicative *lef*s *p47*, *lef-8*, *lef-9*, or *pp31* reduced very late expression to similar levels. In contrast, dsRNA specific to *gp64* had no effect. We concluded that RNA silencing of replicative and nonreplicative *lef*s was sufficient to suppress Ac*M*NPV late gene expression.

Replicative *lef* silencing blocks replication of Ac*M*NPV DNA. To determine the contribution of the *lef*s to virus DNA replication during infection, we silenced each *lef* and monitored virus DNA accumulation by using quantitative real-time PCR. In the absence of dsRNA, newly synthesized Ac*M*NPV DNA was detected in SF21 cells as early as 6 h after infection, whereupon it increased through 12 h and peaked thereafter (Fig. 2A). Verifying the reliability of our PCR assay, this pattern of virus DNA accumulation is typical of L-1 Ac*M*NPV infection of permissive SF21 cells (25, 43). Importantly, the kinetics of viral DNA synthesis in nonpermissive *Drosophila* cells was comparable (Fig. 2B), as described below. By 12 h after infection, dsRNA specific for the replicative *lef*s *ie-1*/*ie-0*, *p143*, *lef-1*, *lef-2*, *lef-3*, *lef-11*, and *dnapol* reduced accumulation of viral DNA by more than 10-fold compared to that by control *egfp* dsRNA (Fig. 3). Similar reductions were observed at 24 and 48 h. In contrast, levels of viral DNA in cells transfected with dsRNAs specific to the nonreplicative *lef*s *p47*, *lef-8*, *lef-9*, or *pp31* were as high or higher than that in cells transfected with dsRNA specific for *egfp* or *gp64*. Virus DNA accumulation in *lef-8*- and *pp31*-silenced cells was reduced at 12 h after

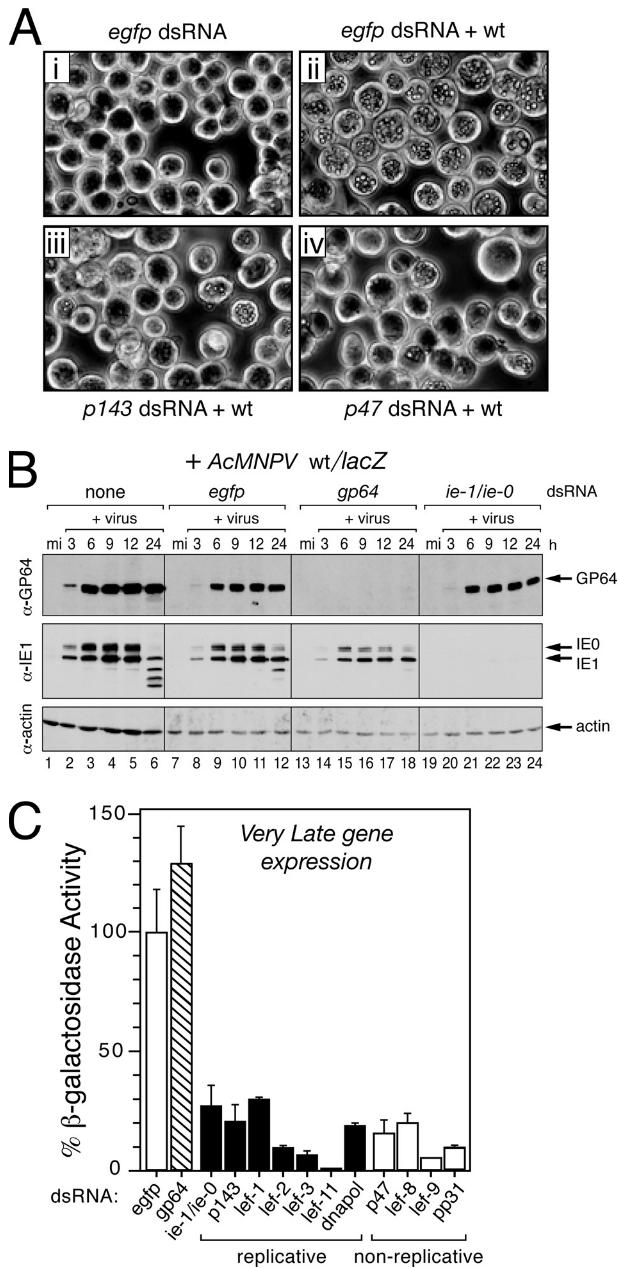

FIG. 1. Silencing of Ac*M*NPV *lef*s blocks very late gene expression. (A) Polyhedra accumulation. SF21 monolayers were transfected with the indicated dsRNAs and inoculated 24 h later with wild-type (+wt) Ac*M*NPV (MOI = 10). Representative photographs (×500 magnification) taken 48 h after infection are shown. (B) Intracellular levels of Ac*M*NPV early proteins. SF21 cells were transfected and then mock infected (mi) or infected with Ac*M*NPV recombinant wt/lacZ as described for panel A. Whole-cell lysates prepared at the indicated times (in hours) after infection were subjected to immunoblot analysis by using anti-GP64 (top), anti-IE1 (middle), and anti-actin (bottom); IE1 and its larger splice variant IE0 were detected by anti-IE1. (C) Very late *polh* gene expression. SF21 cells were transfected with the indicated dsRNAs and infected with wt/lacZ (MOI = 10) in which the *polh* promoter directs *lacZ* expression. Gene expression was quantified by measuring β-galactosidase in cell extracts prepared 48 h after infection. The values reported are the average β-galactosidase activity ± the standard deviation from duplicate infections normalized to that of wt/lacZ-infected cells transfected with control *egfp*-specific dsRNA. The results of a representative experiment are shown.

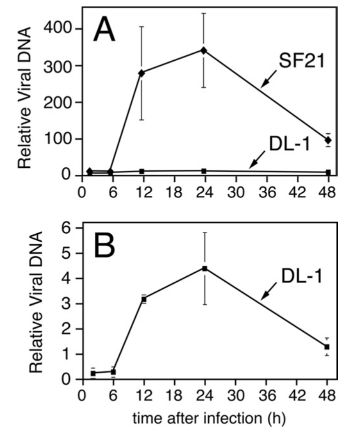

FIG. 2. Kinetics of intracellular Ac*M*NPV DNA accumulation is similar in lepidopteran and dipteran cells. *Spodoptera* SF21 and *Drosophila* DL-1 monolayers were inoculated (MOI = 10) with Ac*M*NPV recombinants wt/lacZ and vP35, respectively; these viruses encode caspase inhibitor *p35* under the control of the early *p35* or *ie-1* promoters, respectively, which prevents apoptosis of the infected cell. At the indicated times after inoculation, cells were collected, lysed, and extracted for total DNA. (A) Intracellular Ac*M*NPV DNA was quantified by using real-time PCR. Values reported are the quantities of virus DNA ± the standard deviation at the indicated times for triplicate plates and normalized to that detected in mock-infected cells. Nucleic acid samples were normalized for cell equivalents by measuring SF21 and DL-1 genomic DNA content by using the *Spodoptera sfiap* and *Drosophila* β-*actin* genes, respectively, as standards. (B) High-resolution depiction of panel A to highlight the lower levels of Ac*M*NPV DNA accumulation in DL-1 cells.

infection, but not 24 or 48 h (Fig. 3). These findings were confirmed in *Drosophila* cells, whereupon *lef-8* and *pp31* silencing had no effect on Ac*M*NPV DNA levels (see below). We concluded that silencing the replicative *lef*s blocked or reduced virus DNA accumulation and thus established their role in Ac*M*NPV DNA replication during infection. Moreover, the nonreplicative *lef*s *p47*, *lef-8*, *lef-9*, and *pp31* have little or no effect on virus DNA synthesis.

Replicative but not nonreplicative *lef*s contribute to Ac*M*NPV-mediated inhibition of host protein synthesis. To verify the effects of gene silencing on virus replicative events, we monitored the temporal synthesis of viral proteins. Early, late, and very late phases of Ac*M*NPV protein synthesis are readily distinguished by radiolabeling (8, 9, 19, 33). We therefore labeled dsRNA-transfected cells with [^{35}S]methionine-cysteine for 1-h periods after infection with Ac*M*NPV wt/lacZ. As expected, dsRNA specific to *p143*, *lef-3*, *p47*, and *pp31* ablated the protein product of each gene as indicated by the absence of these proteins at 8 and 12 h after infection (Fig. 4, lanes 11 to 16 and lanes 19 to 24). Upon silencing these individual genes, there was little or no effect on the early pattern (4 and 8 h) of virus and host proteins compared to that of cells transfected with control *egfp* dsRNA (lanes 1 to 16). In contrast, *p143*- and *lef-3*-silenced cells synthesized no detectable late (24 h) or very late (48 h) viral proteins (lanes 29 to 30 and lanes 37 and 38). Rather, these cells continued to synthesize several early viral proteins then. Upon silencing the late RNA polymerase subunit gene *p47* or nonreplicative *pp31*, late and very late virus proteins were reduced or eliminated (lanes 31 to 32 and lanes 39 and 40). By comparison, untreated or *egfp* dsRNA-treated

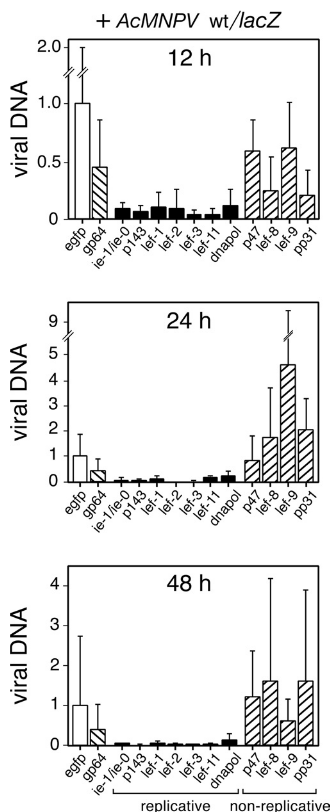

FIG. 3. Ac*M*NPV DNA synthesis is blocked upon silencing of the replicative *lef*s. SF21 monolayers were transfected with the indicated dsRNAs and infected 24 h later with Ac*M*NPV wt/lacZ (MOI = 10). At 12, 24, and 48 h after infection, the cells were collected, lysed, and extracted for total DNA. Ac*M*NPV DNA levels were quantified by real-time PCR as described in Fig. 2. Values are reported as the level of viral DNA ± the standard deviation from six different plates compared to that of control *egfp* dsRNA-transfected, wt/lacZ-infected cells. Solid and cross-hatched bars depict values obtained upon transfection of dsRNA specific for replicative and nonreplicative *lef*s, respectively.

cells exhibited normal or near normal synthesis of late and very late proteins (lanes 27 to 28 and lanes 35 and 36), including *polh* promoter-directed β-galactosidase. We concluded that silencing the replicative and nonreplicative *lef*s was sufficient to block the transition from early to late phases of infection.

At late times after Ac*M*NPV infection, host protein synthesis declines dramatically, whereas late viral proteins accelerate. Although the mechanisms and baculovirus genes responsible for this host shutdown are unknown (for a review, see reference 49), the response is common among diverse viruses. Ac*M*NPV inhibition of host protein synthesis was readily apparent in untreated and *egfp* dsRNA-transfected cells by 24 and 48 h (Fig. 4, lanes 27 to 28 and lanes 35 and 36). In contrast, cells transfected with dsRNA specific to replicative *lef*s *p143* and *lef-3* exhibited near-normal levels of host protein synthesis, as indicated by efficient radiolabeling of nonvirus proteins at the same times (lanes 29 to 30 and lanes 37 and 38). Silencing the envelope fusion protein gene *gp64*, another early gene, had no effect on the inhibition of host protein synthesis (data not shown). Thus, silencing these replicative *lef*s was sufficient to suppress virus-mediated inhibition of host protein synthesis. In contrast, host protein synthesis inhibition was unaffected upon silencing the nonreplicative *lef*s *p47* and *pp31* (Fig. 4, lanes 31 to 32 and lanes 39 and 40). By 48 h, few if any host proteins were radiolabeled in either *p47*- or *pp31*-silenced cells (lanes 39 to 40) compared to *p143*- and *lef-3*-silenced cells (lanes 37 and 38). Thus, these nonreplicative *lef*s failed to contribute to Ac*M*NPV-mediated shutoff of host protein synthesis. We concluded that whereas both replicative and nonreplicative *lef*s are required for the transition to late stages of infection, only the replicative *lef*s are required for virus-mediated translational arrest. This finding is consistent with viral DNA replication acting as the signal for host protein synthesis shutdown.

Replicative but not nonreplicative *lef*s are required for Ac*M*NPV-induced apoptosis. Ac*M*NPV-mediated apoptotic signaling requires *ie-1* (46). However, it was unclear whether the proapoptotic activity of *ie-1* is due to its promotion of downstream virus events, including virus DNA replication, late gene expression, or both. Here, by selectively silencing components of the Ac*M*NPV late stage RNA polymerase, it was possible to independently evaluate the contribution of virus DNA replication and late virus gene expression to apoptotic signaling.

To this end, we monitored the level of virus-induced apoptosis in RNA-silenced SF21 cells by using the Ac*M*NPV mutant vΔ35K/lacZ ($p35^-$ $polh^-$ $lacZ^+$), which lacks the caspase inhibitor *p35* and thus cannot prevent caspase-mediated apoptosis. Transfection of control *egfp* dsRNA had no effect on the capacity of vΔ35K/lacZ to trigger widespread apoptosis in SF21 cells (Fig. 5A, compare panels i and ii). By 24 h after infection, apoptotic blebbing and cytolysis encompassed >90% of the culture, which was comparable to that in the absence of dsRNA (46). Proteolytic processing of the *Spodoptera* effector caspase, Sf-caspase-1, and the usual appearance of intracellular caspase activity confirmed that cell death was by apoptosis (data not shown). In contrast, *p143*-specific dsRNA prevented vΔ35K/lacZ-induced apoptosis (Fig. 5Aiii). Quantitation indicated that >60% of these cells survived through 24 h (Fig. 5B), which was comparable to that of cells treated with *ie-1*/*ie-0* dsRNA (46). Because *p143*-silenced cells synthesized early viral proteins (except P143) at normal levels (Fig. 4), the lack of apoptosis was not due to inefficient infection. Virus-induced apoptosis was also reduced upon silencing the replicative *lef*s *lef-3*, *lef-2*, *dnapol*, and *lef-11*, in order of decreasing effectiveness (Fig. 5B); cell survival was 2.5- to 10-fold higher than that of cells transfected with control *egfp* or *gp64* dsRNA. Interestingly, *lef-1* had the smallest contribution to virus-induced apoptosis in SF21 cells (Fig. 5B). Nonetheless, *lef-1* was essential for virus-induced apoptosis in *Drosophila* cells (see below). We concluded that replicative *lef*s are required for Ac*M*NPV-induced apoptosis but that they differ in their proapoptotic activities. It is noteworthy that when silenced individually none of the replicative *lef*s restored survival of vΔ35K/lacZ-infected cells to 100%. We attributed this effect to either incomplete silencing that allowed very low levels of DNA replication or to other early proapoptotic events not associated with virus DNA replication, as suggested previously (25).

When SF21 cells were transfected with dsRNA specific to nonreplicative *lefs* *p47*, *lef-8*, *lef-9*, or *pp31*, they remained sensitive to Ac*M*NPV-induced apoptosis (Fig. 5B). For each of the nonreplicative *lef*s, <5% of the cells survived infection with

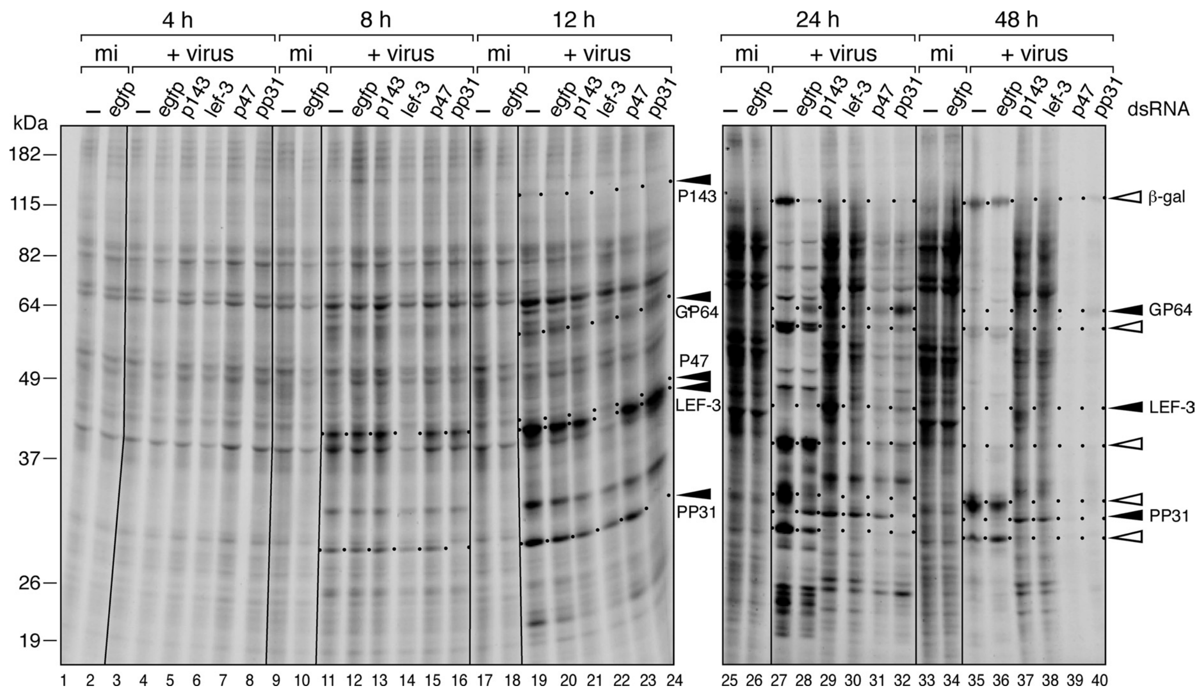

FIG. 4. Ac*M*NPV-mediated inhibition of host protein synthesis is prevented upon silencing of replicative *lef*s. SF21 monolayers were transfected with the indicated dsRNAs and infected 24 h later with Ac*M*NPV wt/lacZ (MOI = 10). The cells were radiolabeled for 1 h with [^{35}S]methionine-cysteine, collected at the indicated times (in hours), and lysed. Protein samples from equal cell numbers were subjected to SDS-PAGE and autoradiography. Early and late/very late virus proteins are denoted by closed and open arrows, respectively; unlabeled arrows denote unknown late virus proteins. Protein size standards (in kilodaltons) are indicated to the left. The two autoradiographs (4 to 12 h and 24 to 48 h) represent two contiguous gels of a representative experiment.

vΔ35K/lacZ. Virus-induced apoptotic blebbing and cytolysis were extensive, as typified by *p47*-silenced cells (Fig. 5Aiv). Because the conditions for silencing the nonreplicative *lef*s were identical to those that prevented late gene expression (Fig. 1 and 4), we concluded that silencing was achieved here. These findings indicated that the nonreplicative *lef*s are not required for virus-induced apoptosis and therefore suggested for the first time that late virus multiplicative events contribute little to apoptotic signaling.

Replicative *lef*s are required for Ac*M*NPV DNA replication in nonpermissive dipteran cells. To further test the role of viral DNA replication in triggering apoptosis, we determined the effect of *lef* ablation on Ac*M*NPV-induced apoptosis of DL-1 cells, a *Drosophila* cell line that is highly responsive to RNA-mediated gene silencing (27, 46, 47). DL-1 cells fail to support productive infection by Ac*M*NPV but are highly sensitive to apoptosis induced by inoculation of Ac*M*NPV mutants that lack apoptotic suppressors (27, 34, 37, 60). Thus, early virus events that include DNA replication may be sufficient to trigger apoptosis (46). To define the kinetics of Ac*M*NPV DNA replication in DL-1 cells, we quantified intracellular viral DNA. Real-time PCR detected virus DNA by 6 h after inoculation (Fig. 2B). This DNA increased through 12 and 24 h and then declined. At peak accumulation, virus DNA was ~80-fold lower than that of permissive SF21 cells on a per-cell basis (Fig. 2A). Nonetheless, the kinetics of virus DNA accumulation was strikingly similar in both cell lines and argued that viral DNA synthesis is an active process in *Drosophila* cells.

Active DNA synthesis was confirmed by demonstrating that replicative *lef* silencing decreased Ac*M*NPV DNA accumulation in DL-1 cells (Fig. 6). Transfection with dsRNA specific to the replicative *lef*s *ie-1*/*ie-0*, *p143*, *lef-1*, *lef-2*, *lef-3*, and *dnapol* reduced virus DNA accumulation at 12, 24, and 48 h after inoculation by ca. 10- to 30-fold compared to that in the presence of *egfp* dsRNA. Only *lef-11* dsRNA failed to affect virus DNA accumulation (Fig. 6). In contrast to the replicative *lef*s, dsRNAs specific to the nonreplicative *lef*s *p47*, *lef-8*, *lef-9*, and *pp31* had no effect. Interestingly, virus DNA accumulation was highest in *gp64*-silenced cells. We concluded that the replicative *lef*s, with the exception of *lef-11*, are required for virus DNA synthesis in *Drosophila* cells. Conversely, the nonreplicative *lef*s are dispensable.

Replicative *lef*s are required for Ac*M*NPV-induced apoptosis in DL-1 cells. Having established that replicative *lef*s are required for virus DNA synthesis, we next tested their contribution to virus-induced apoptosis. To this end, we used Ac*M*NPV recombinant vOpIAP, which lacks *p35* but encodes Op-*iap3* that fails to block apoptosis in *Drosophila* (27, 58, 60). Op-*iap3* prevents apoptosis in permissive SF21 cells and thus facilitated production of high-titered vOpIAP stocks used here. As expected, vOpIAP caused widespread apoptosis in DL-l cells transfected with control *egfp* dsRNA (Fig. 7Aii); by 24 h after inoculation, >75% of these cells underwent membrane blebbing and cytolysis, which is the result of *Drosophila* caspase activation (46). The absence of cytolysis of mock-infected cells transfected with the same dsRNA indicated that apoptosis was virus mediated (Fig. 7Ai). In contrast, transfection with replicative *p143*-specific dsRNA sup-

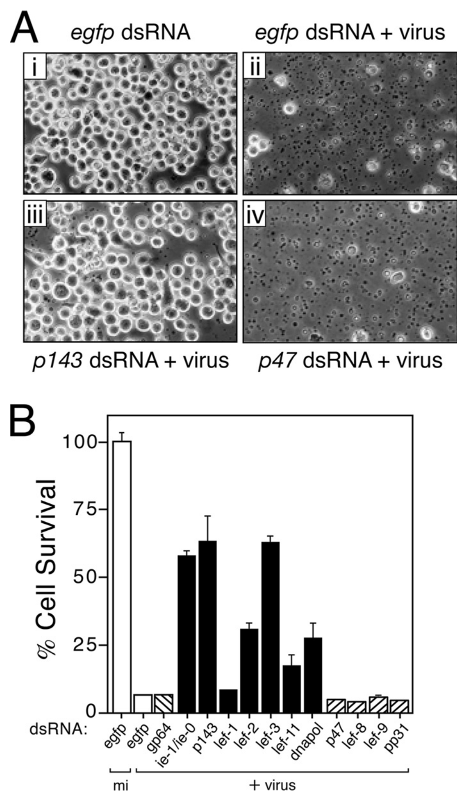

FIG. 5. Ac*M*NPV-induced apoptosis is blocked upon silencing of replicative but not nonreplicative *lef*s. (A) Apoptotic cytolysis. SF21 monolayers were transfected with the indicated dsRNAs and inoculated (+virus) 24 h later with apoptosis-inducing Ac*M*NPV *p35*-deletion mutant vΔ35K/lacZ (MOI = 10). Representative photographs (×250 magnification) taken 26 h after infection are shown. (B) Cell survival. Intact SF21 cells that were mock-infected (mi) or infected (+virus) 26 h earlier with vΔ35K/lacZ as described for panel A were counted by using computer-aided microscopy. Values shown represent the percent ± the standard deviation of surviving cells averaged for three independent plates and compared to that of mock-infected, *egfp* dsRNA-transfected cells. The results of a representative experiment are shown.

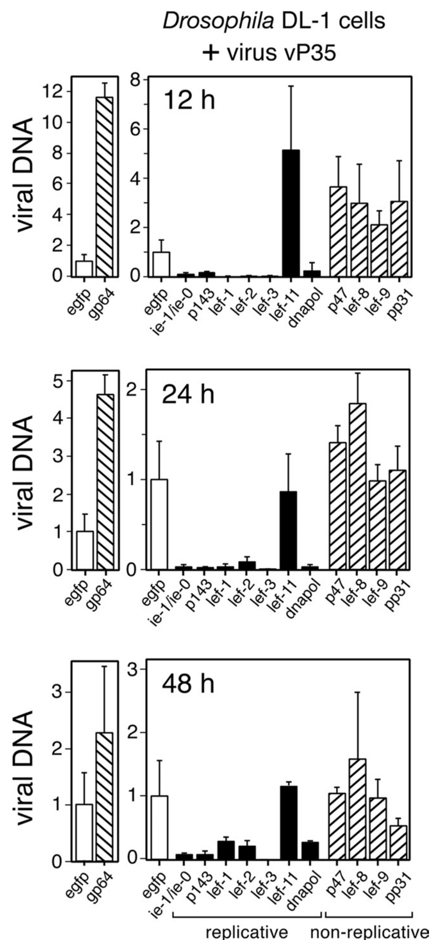

FIG. 6. Ac*M*NPV DNA synthesis in DL-1 cells is blocked upon silencing of replicative *lef*s. DL-1 monolayers were transfected with the indicated dsRNAs and infected 24 h later with Ac*M*NPV vP35 (MOI = 10). Intracellular virus DNA was quantified by real-time PCR as described in Fig. 2. Values are reported as the level of viral DNA ± the standard deviation from four different plates relative to that of control *egfp* dsRNA-transfected, vP35-infected cells. Solid and cross-hatched bars depict values obtained upon transfection of dsRNA specific for replicative and nonreplicative *lef*s, respectively.

pressed virus-induced apoptosis (Fig. 7Aiii); >75% of these cells survived virus inoculation through 24 h, which was a level comparable to that of *ie-1*-silenced cells (Fig. 7B). Likewise, dsRNA specific to replicative *lef-1*, *lef-2*, *lef-3*, or *dnapol* increased cell survival. Only *lef-11*-specific dsRNA failed to suppress apoptosis (Fig. 7B). Because only those replicative *lef*s that contributed to virus-induced apoptosis were also necessary for Ac*M*NPV DNA synthesis (Fig. 6), we concluded that viral DNA synthesis triggers apoptosis in *Drosophila* cells.

The Ac*M*NPV nonreplicative *lefs p47*, *lef-8*, *lef-9*, and *pp31* had little or no effect on vOpIAP-induced apoptosis of DL-1 cells (Fig. 7B). The reduced survival of these cells was comparable to those transfected with control *egfp* or *gp64* dsRNAs. Moreover, the level of virus-induced membrane blebbing and cytolysis was comparable to that of control *egfp* dsRNA-treated cells (Fig. 7A). Although these findings suggested that the nonreplicative *lef*s fail to contribute to virus-induced apoptosis, it is relevant to note that Ac*M*NPV late and very late gene expression is greatly reduced or absent in DL-1 cells (37). Thus, in the absence of late multiplicative events it is unlikely that silencing of nonreplicative *lef*s would have an observed effect.

DISCUSSION

By using RNA silencing to selectively knock down expression of essential genes required for baculovirus multiplication, we report here that Ac*M*NPV replicative *lef*s, but not nonreplicative *lef*s, are required to trigger apoptosis in cells derived from different orders of insects (*Lepidoptera* and *Diptera*). Our study indicates that baculovirus DNA replication activities or events, rather than virus late gene expression, are responsible for host apoptosis. Importantly, RNA silencing of replicative but not nonreplicative *lef*s abrogated Ac*M*NPV-induced shutoff of host protein synthesis, suggesting that virus DNA replication also triggers inhibition of host biosynthetic processes during infection. Thus, the shutoff of host protein synthesis and apoptotic signaling may be linked. Our findings are consistent with a model in which baculovirus DNA replication triggers a host cell response, like that occurring during DNA damage or cell cycle perturbations, which cause translational arrest and

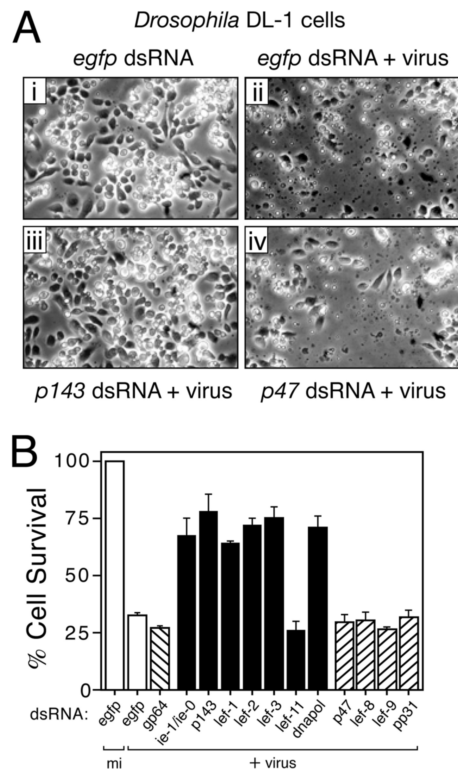

FIG. 7. Ac*M*NPV-induced apoptosis of DL-1 cells is blocked upon silencing of replicative but not nonreplicative *lef*s. (A) Apoptotic cytolysis. DL-1 monolayers were transfected with the indicated dsRNAs and inoculated (+virus) 24 h later with Ac*M*NPV recombinant vOpIAP (MOI = 10), which lacks *p35* and causes vigorous apoptosis in DL-1 cells (27, 58, 60). Representative photographs (×250 magnification) taken 24 h after infection are shown. (B) Cell survival. Intact DL-1 cells that were mock-infected (mi) or infected (+virus) 24 h earlier with vOpIAP as described for panel A were counted by using computer-aided microscopy. Values shown represent the percent ± the standard deviation of surviving cells averaged for three independent plates and normalized to that of mock-infected, *egfp* dsRNA-transfected cells. The results of a representative experiment are shown.

apoptosis in vertebrates. Thus, pathways for sensing virus invasion and triggering apoptosis appear to be conserved between insects and mammals.

Different roles for Ac*M*NPV *lef*s during permissive infection. The *lef*s were originally defined by transient-transfection assays as factors required for baculovirus late gene expression and were subsequently assigned as replicative and nonreplicative on the basis of their role in transient DNA replication assays (41). As determined by gene-specific RNA silencing, we report here that Ac*M*NPV *lef*s *ie-1*/*ie-0*, *p143*, *lef-1*, *lef-2*, *lef-3*, *lef-11*, and *dnapol* (Table 1) contribute to virus DNA replication and late gene expression during permissive Ac*M*NPV infection (Fig. 1 and 3). Our data confirm the critical roles of *ie-1*/*ie-0*, *p143*, *lef-11*, and *dnapol* as demonstrated by conditional lethal mutations or gene knockouts (31, 32, 48, 53). The roles of *lef-1*, *lef-2*, and *lef-3* for DNA replication, and thus late gene expression, during infection are established here. We also demonstrated that IE1 is required for Ac*M*NPV DNA replication during infection (Fig. 3 and 6). In addition to its transactivation potential for replicative genes, IE1 likely has a direct role in Ac*M*NPV DNA synthesis (D. Taggart and P. Friesen, unpublished data). Ac*M*NPV nonreplicative *lef*s had little or no effect in virus DNA replication but were required for late gene expression (Fig. 1 and 3). Thus, our study confirmed the critical roles of *p47* and *pp31* for late gene expression (3, 59) and demonstrated definitive roles for RNA polymerase subunits *lef-8* and *lef-9* during infection.

RNA silencing is effective because it selectively ablates gene products and thereby abolishes gene function. Here, we verified the loss of viral proteins IE1, IE0, and EFP GP64 by immunoblot analysis of silenced cells (Fig. 1B). Ablation of P143, LEF-3, P47, and PP31 was confirmed by protein radiolabeling during infection (Fig. 4). Due to low-level synthesis or antisera unavailability, we have not confirmed the depletion of LEF-1, -2, -8, -9, -11, or DNA polymerase. However, because of the striking inhibition of Ac*M*NPV multiplicative processes effected by dsRNAs specific to their genes (Fig. 1, 3, and 6), we concluded that significant silencing was accomplished.

Induction of apoptosis by Ac*M*NPV DNA replication. Previous studies have suggested that baculovirus DNA synthesis or late gene expression, which is dependent on virus DNA replication, triggers apoptosis during infection (10, 25). By preventing late gene expression through silencing nonreplicative *lef*s comprising the Ac*M*NPV late stage RNA polymerase (*p47*, *lef-8*, and *lef-9*), we demonstrated here that late gene expression is not required for virus-induced apoptosis of *Spodoptera* cells (Fig. 4). Conversely, ablation of six of seven Ac*M*NPV replicative *lef*s, including *ie-1*/*ie-0*, *p143*, *lef-2*, *lef-3*, *lef-11*, and *dnapol*, arrested virus-induced apoptosis. Because silencing of multiple, independent targets required for virus DNA replication had the identical effect of suppressing virus-induced apoptosis, it is likely that one or more activities constituting the DNA replication process is responsible for triggering apoptosis. Not ruled out is the possibility that a late virus gene, expressed independent of the late-stage RNA polymerase but dependent on virus DNA replication, is responsible for apoptosis. However, this possibility is unlikely because dsRNA-mediated ablation of *pp31* was as ineffective in preventing Ac*M*NPV-induced apoptosis as that of the late RNA polymerase subunits (Fig. 4). The *pp31* product is a DNA-binding protein not associated with the late RNA polymerase but required for late gene expression (41).

It is noteworthy that among the replicative *lef*s, ablation of *ie-1*/*ie-0*, *p143*, or *lef-3* had the strongest suppressive effect on Ac*M*NPV-induced apoptosis (Fig. 4). This difference could be attributed to enhanced sensitivity of these *lef*s to RNA silencing. Alternatively, it is possible that the replicative *lef*s differ in their proapoptotic activities. In particular, IE1 may upregulate expression of the other replicative *lef*s through its transactivator properties, as well as initiate viral DNA synthesis by direct binding to origins of virus DNA replication and recruiting factors of the replication complex (D. Taggart and P. Friesen, unpublished data). LEF-3 and P143, which are interdependent for nuclear import, may also provide required early functions for DNA synthesis, including virus DNA unwinding and formation of the replication complex (reviewed in reference 52). Thus, ablation of any one of these replicative factors may have a potent negative effect at the earliest stage of apoptotic signaling. Interestingly, *lef-1* was required for Ac*M*NPV DNA synthesis, but not virus-induced apoptosis in *Spodoptera* cells

(Fig. 1 and 4). In contrast, *lef-1* was required for both activities in *Drosophila* cells. Although additional studies are required to define the mechanisms by which *lef-1* and the other *lef*s promote virus DNA replication and trigger apoptosis (see below), this finding strengthens the premise that the replicative *lef*s have nonequivalent proapoptotic activities.

Ac*M*NPV DNA replication-induced apoptosis in *Drosophila*. The proapoptotic activities of the replicative *lef*s were confirmed by our finding that they are also required for Ac*M*NPV-induced apoptosis in *Drosophila* DL-1 cells (Fig. 7). Although dipteran DL-1 cells are nonpermissive for Ac*M*NPV, inoculation with mutants that lack antiapoptotic genes causes widespread apoptosis that is caspase dependent (27, 37, 58, 60). Quantitative PCR revealed that although total accumulation of Ac*M*NPV DNA is significantly lower than that in permissive *Spodoptera* cells, the kinetics of viral DNA synthesis were comparable (Fig. 2). With the exception of *lef-11*, all of the replicative *lef*s were required for viral DNA synthesis in DL-1 cells (Fig. 6); the nonreplicative *lef*s were dispensable. We concluded that the mechanics of Ac*M*NPV DNA synthesis are similar in both *Drosophila* and *Spodoptera*. Each of the replicative *lef*s required for viral DNA synthesis also contributed to Ac*M*NPV-induced apoptosis (Fig. 7). This finding suggested that Ac*M*NPV triggers apoptosis by comparable mechanisms in dipteran and lepidopteran species. Thus, the pathway by which insects detect DNA virus entry and respond by apoptosis may be conserved. Our study also endorses the use of *Drosophila* as an advantageous model system for defining mechanisms of DNA virus-induced apoptosis.

Host protein synthesis arrest by Ac*M*NPV replicative *lef*s. Inhibition of host protein synthesis is common among viruses (reviewed in references 11 and 45). Baculoviruses induce a dramatic but poorly understood shutoff of host protein synthesis that parallels late and very late gene expression (49). It is unclear whether this shutoff is a virus-based strategy to selectively facilitate virus protein synthesis or whether it is a host-mediated response to establish an antiviral state. We discovered here that silencing Ac*M*NPV replicative *lef*s prevented virus-induced translational arrest of host proteins (Fig. 4 and data not shown); nonreplicative *lef*s had no effect. Because ablation of independent replicative *lef* targets had the same effect, we concluded that virus DNA replication activities rather than late gene functions trigger the inhibitory response.

Our finding that host translational arrest and apoptosis are triggered by the same baculovirus replication events raises the possibility that both processes are linked and represent host responses. Shutoff of host protein synthesis can trigger apoptosis by blocking replenishment of short-lived antiapoptotic proteins, including the invertebrate IAP proteins that are required for cell survival (18, 24). For example, nodavirus-mediated depletion of the principal IAP of *Drosophila*, DIAP1, can trigger apoptosis of *Drosophila* DL-1 cells upon infection (47). Our preliminary studies indicated that DIAP1 and SfIAP, the *Spodoptera* cellular IAP, are also depleted upon Ac*M*NPV infection (R. Cerio, K. Schultz, Rianna Vandergaast, and P. Friesen, unpublished data). In mammals, protein synthesis inhibition often precedes cell cycle arrest and apoptosis that is mediated by tumor suppressor p53, which is activated upon virus infection or DNA damage (12). In *Drosophila*, DNA damage-induced activation of Dmp53 upregulates proapoptotic genes, including *reaper*, that antagonize DIAP1 antiapoptotic activity and inhibit global protein synthesis (reviewed in references 50 and 54). In *Spodoptera*, baculovirus DNA replication-triggered protein synthesis arrest may provide supplementary contributions toward apoptosis. Thus, analogous to the large herpesviruses in which multiple genes or events cause apoptosis (17, 38), baculoviruses may also present multiple apoptotic triggers (25). Further study is required to distinguish the potential roles of host lepidopteran genes in translational arrest and apoptosis during baculovirus infection.

Mechanisms of baculovirus DNA replication-induced apoptosis. The induction of unscheduled DNA synthesis during infection or the detection of replicating viral DNA as damaged DNA are potent proapoptotic signals in vertebrates (reviewed in references 29, 30, and 57). Because the replication of DNA virus genomes often requires active host DNA synthetic machinery, pathogens such as adenovirus, simian virus 40, and human papillomavirus induce cell cycle progression that pushes cells into S phase and as a consequence triggers the DNA damage apoptosis pathways. Thus, to promote multiplication, these viruses encode factors that inactivate p53 and block apoptosis (reviewed in references 29, 30, and 57).

DNA damage and unscheduled DNA synthesis are sufficient to trigger apoptosis in invertebrates, including *Drosophila* (2, 36, 54). The highly conserved DNA repair machinery of *Drosophila* is capable of recognizing DNA breaks and single-stranded DNA, which activates Dmp53 and upregulates proapoptotic factors including Reaper (2, 36). Representing various intermediates during virus DNA replication, double-stranded DNA ends or stretches of single-stranded DNA are recognized as damaged DNA (30). It is unknown how the large (>120 kb), circular DNA genome of baculoviruses is replicated (reviewed in references 39 and 52). Several replication strategies have been proposed, including rolling circle and recombination-mediated mechanisms. Each of these pathways involves the generation of replication intermediates that should be recognized by the host repair machinery as damaged DNA and thereby trigger cell cycle arrest and apoptosis. Indeed, Ac*M*NPV replication causes cell cycle perturbations (1, 23, 42). Thus, the pathways by which apoptosis are triggered in DNA virus-infected cells may be conserved between insects and mammals. Additional studies should uncover both novel and conserved mechanisms by which baculovirus DNA replication is detected by the host insect cell and triggers apoptosis.

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