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Apoptosis Reduces both the In Vitro Replication and the In Vivo Infectivity of a Baculovirus

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Apoptotic programmed cell death occurs when the insect cell line SF-21, derived from *Spodoptera frugiperda*, is infected with mutants of the baculovirus *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) which lack a functional *p35* gene. However, infection of the *Trichoplusia ni* TN-368 cell line with *p35* mutants does not result in apoptosis (R. Clem, M. Fechheimer, and L. Miller, Science 254:1388–1390, 1991). We have examined the effect of apoptosis on Ac*M*NPV infections in cell lines and larvae of these two insect species. Production of viral progeny was significantly lower in SF-21 cells infected with *p35* mutants than in cells infected with wild-type (wt) or revertant viruses. Viral gene expression was abnormal in SF-21 cells infected with *p35* mutants; there was a delay in the transcription and translation of early and late viral genes, a lack of expression of very late genes, and a total cessation of protein synthesis late in the apoptotic process. In vivo analysis revealed that the dose of budded virus required for 50% lethality in *S. frugiperda* larvae was approximately 1,000-fold higher for *p35* mutants than for wt or revertant viruses. In contrast, the replication and infectivity of *p35* mutant viruses was equivalent to that of wt Ac*M*NPV during infection of both TN-368 cells and *T. ni* larvae. Thus, the data indicate that a host apoptotic response provides protection against viral infection at the organismal level and that the *p35* gene constitutes a host range determinant for Ac*M*NPV infection.

Apoptosis, a distinctive type of programmed cell death, was first described in 1972 by Kerr et al. (22) and is now widely recognized as being vital to a diversity of biological processes, including embryonic development, tissue homeostasis, tumorigenesis, and the lysis of virally infected cells by cytotoxic T lymphotytes (42). In addition to being important in organismal and cellular functions, apoptosis also appears to be an important factor in the replication strategies of a wide variety of eukaryotic viruses. A number of disparate viruses are known to trigger apoptosis during infection (14, 20, 24a, 28, 40), and members of at least three different virus families, *Herpesviridae*, *Adenoviridae*, and *Baculoviridae*, possess genes which can prevent apoptotic cell death of their host cells (2, 3, 8, 15, 43). Inhibition of apoptosis has also been correlated with viral latency (13a) and persistence (24a). The ability to block premature death of the host cell should provide significant advantages for most obligate intracellular parasites, although the extent of these advantages for lytic viruses has not been fully explored.

Apoptosis occurs upon infection of either the *Spodoptera frugiperda* SF-21 (3, 16) or the *Bombyx mori* BmN-4 (3) cell line with *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) mutants lacking a functional *p35* gene. In the case of SF-21 cells, the majority of the cells undergo apoptosis within 12 to 24 h postinfection (p.i.), and no occlusion bodies are formed (3). In contrast, infection of *Trichoplusia ni* TN-368 cells with *p35* mutants results in normal virus replication, including occlusion body production, during the very late phase of infection between 24 to 70 h p.i. (3, 16). Both the SF-21 and TN-368 cell lines are fully permissive for wild-type (wt) Ac*M*NPV replication, whereas the BmN-4 cell line does not normally support productive Ac*M*NPV replication, although significant expression from all three temporal classes of viral promoters is observed (30). In addition, the *p35*-homologous gene from the baculovirus *B. mori* nuclear polyhedrosis virus (BmNPV) also appears to be involved in preventing apoptosis in BmN cells, as infection with BmNPV mutants lacking the BmNPV *p35*-homologous gene results in a mixed phenotype, with some cells becoming apoptotic and others supporting full virus replication (21). However, although differences in the response of various cell lines to *p35* mutant infection have been observed (3, 16, 21), it is not known whether these differences are species specific or cell line specific.

The mechanism by which P35 acts in blocking apoptosis is not clear, although insights into its function are beginning to emerge. Infection of SF-21 cells with wt Ac*M*NPV but not *p35* mutant viruses is able to block apoptosis triggered by a nonviral signal, actinomycin D, suggesting that P35 (possibly in conjunction with other viral gene products) is able to interact directly in the host apoptotic pathway rather than acting by preventing viral triggering of apoptosis (8). However, if actinomycin D is added to the cells prior to 5 h p.i., apoptosis results (8), indicating that viral gene expression is required for inhibition of cell death. Consistent with a role early in the infection process, the *p35* gene is transcribed at both early and late times in infection (10, 11, 19, 31). The predicted sequence of the P35 polypeptide (11) currently provides little or no help in understanding its function, although the C terminus of P35, including the last 12 amino acids, is known to be essential for function (16).

P35 is not the only baculovirus gene product able to block Ac*M*NPV- or actinomycin D-triggered apoptosis; a second gene encoding a gene product with a zinc finger-like motif was identified in the genome of *Cydia pomonella* granulosis virus (CpGV) as a gene capable of blocking apoptosis triggered by infection with *p35* mutants of Ac*M*NPV (8). Effectively this gene, known as *iap* (for inhibitor of apoptosis), can replace *p35* function in blocking apoptosis in SF-21 cells. A homolog of *iap* is also found in the Ac*M*NPV

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genome, but the AcMNPV *iap*-homologous gene (Ac-*iap*) is unable to block apoptosis in the absence of *p35* (8). It may be that both Ac-IAP and P35 are required and interact to prevent apoptosis during AcMNPV infection of SF-21 cells; alternatively, Ac-IAP may have evolved a different function.

The role of apoptosis in the control of virus infection by cytotoxic T lymphocytes in vertebrates has led to the suggestion that apoptosis may have originally evolved as a defense response to viral infection in primitive animals lacking a humoral immune system (6, 27). The observation that apoptosis occurs in insect cells infected with *p35* mutants of AcMNPV provides an ideal model with which to test this hypothesis and also to explore the impact that apoptosis may have on viral infection in the absence of an antibody response. Very little is known concerning insect defenses against virus infection; although insect hemocytes can provide cell-mediated immunity to bacterial pathogens through phagocytosis or encapsulation (13), neither cell-mediated nor humoral immunity has been demonstrated against baculovirus infection in insects.

In a recent report, Hershberger et al. showed that disruption of *p35* results in reduced yields of progeny budded virus (BV) and decreased synthesis of late viral proteins in SF-21 but not in TN-368 cells (16). Here we confirm and extend the in vitro results of Hershberger et al. and further analyze the replication and infectivity of AcMNPV *p35* mutants in insect larvae. The infectivities of wt AcMNPV and the *p35* mutants were similar in *T. ni* larvae, and replication of these viruses, including the expression of selected viral transcripts and viral proteins, was normal in TN-368 cells. In *S. frugiperda* larvae and SF-21 cells, however, apoptosis had an adverse effect: *p35* mutants were significantly less infectious in vivo and were impaired in their replication, transcription, and protein synthesis in SF-21 cells. This finding represents the first experimental evidence at the organismal level that apoptosis provides host protection against viral infection and supports the hypothesis that apoptosis may have evolved as an antiviral response in primitive animals. The ability to block such a response also appears to be an important host range determinant in baculovirus infections. Finally, a delay in viral gene expression was observed in *p35* mutant-infected SF-21 cells, suggesting a role for P35 in the timely expression of early viral genes involved in blocking the apoptotic response of the cell.

MATERIALS AND METHODS

Viruses, cell lines, and insects. Wild-type (L-1 strain) AcMNPV (24) and the *p35*-revertant viruses vAnhHK5 and vP35ZRS were propagated in IPLB-SF-21 (SF-21) cells (41) by previously described methods (35). The AcMNPV *p35* mutants, annihilator (vAcAnh) and vP35Z (3), were propagated in TN-368 cells (17). BV titers for all of the viruses were determined by plaque assay using TN-368 cells as described previously (35). SF-21 and TN-368 cells were maintained at 27°C in TC-100 medium (GIBCO BRL Laboratories, Gaithersburg, Md.) supplemented with 10% fetal bovine serum (Intergen, Purchase, N.Y.) and 0.26% tryptose broth. *S. frugiperda* and *T. ni* eggs were kindly provided by W. Deryck Perkins (Agricultural Research Service, U.S. Department of Agriculture, Tifton, Ga.) and Beth Gray (Abbott Laboratories, Chicago, Ill.), respectively, and the insects were reared in individual cups on artificial diet (35) at 27°C under a 14-h/10-h light/dark cycle.

Construction of revertant viruses. vAnhHK5 was constructed by calcium phosphate cotransfection (35) of SF-21 cells with vAcAnh DNA and the lambda clone HK5 (37), which contains wt AcMNPV sequences from approximately 80 to 91 map units (including *p35*), and screening for viruses with an occlusion-positive plaque phenotype. vP35ZRS was constructed similarly except that vP35Z DNA and plasmid pRS, containing the *Eco*RI S fragment of wt AcMNPV from 86.8 to 87.9 map units (including *p35*) were used. The construction of both revertant viruses was verified by restriction enzyme analysis and Southern blotting.

Protein pulse labeling. SF-21 or TN-368 cells (10^6 of each) were mock infected with TC-100 or infected with wt AcMNPV, vAcAnh, or vP35Z at a multiplicity of infection (MOI) of 20 PFU per cell. After a 1-h adsorption period, the viral inoculum was removed and replaced with complete TC-100; the time of refeeding was taken as time zero. Two hours before the appropriate time point, the medium was removed and replaced with incomplete TC-100 lacking methionine. Because of the propensity for *p35* mutant-infected SF-21 cells and apoptotic bodies to lift off the plate, all of the SF-21 samples were refed by scraping the cells, transferring the cells and the culture supernatant to centrifuge tubes, centrifuging the cells 5 min at 1,000 × *g*, and gently resuspending the pellets in methionine-free TC-100. Following incubation for 1 h, 25 μCi of [^{35}S]methionine (New England Nuclear, Boston, Mass.) was added per plate or tube, and the cells were incubated for an additional hour. The monolayers or cell pellets were then washed and lysed in 50 μl of lysis buffer as described previously (35). The lysates were stored at −80°C until they were analyzed. Five microliters of each lysate was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (23) on 10% polyacrylamide gels and subjected to fluorography as described previously (35).

Primer extensions. SF-21 or TN-368 cells (6×10^6 of each) were mock infected or infected with wt AcMNPV or vP35Z at an MOI of 20 PFU per cell as described above. At 6, 12, 24, and 48 h p.i., the cells were harvested and total RNA was isolated by the guanidinium isothiocyanate method (1). The cells were harvested by scraping, and then both the cells and the culture supernatant were centrifuged as described above to avoid losing any apoptotic bodies. Twenty micrograms of RNA was hybridized to oligonucleotides complementary to the *egt*, capsid, or polyhedrin open reading frame (ORF) (see below) which had been 5′ end labeled with T4 polynucleotide kinase (GIBCO BRL). The oligonucleotide primers were then extended by using Moloney murine leukemia virus reverse transcriptase (GIBCO BRL), and the extended products were analyzed by urea-polyacrylamide gel electrophoresis and autoradiography.

The oligonucleotides used for primer extension were as follows: *egt*, 5′-AGTGCAAGCCAGCAGAG-3′, complementary to the sequence from +26 to +10 of the *egt* ORF (nucleotides 174 to 158 of reference 34); capsid, 5′-CGCC ATACCCACGGG-3′, complementary to the sequence from +27 to +13 of the *vp39* ORF (nucleotides 491 to 477 of reference 39); and polyhedrin, 5′-GGTACGCCCGATGGT GGG-3′, complementary to the sequence from +39 to +22 of the *polh* ORF (18).

Virus growth curves. SF-21 or TN-368 cells (2×10^6 of each) were infected with virus at an MOI of 20 PFU per cell as described above except that after removal of the virus inoculum, the cell monolayers were washed two times with TC-100 and then refed with 5 ml of TC-100. Immediately and at 12, 24, and 48 h p.i., 0.5 ml of the culture medium was removed and stored at 4°C until determination of titers. Samples were titered by plaque assay using TN-368 cells.

Larval bioassays. Various doses of BV (as determined by plaque assay using TN-368 cells) were injected into the hemocoel of developmentally staged larvae (25 per dose) within 24 h of molting into the penultimate larval instar (fifth-instar *S. frugiperda* or fourth-instar *T. ni*). Complete TC-100 was used to dilute the BV samples. Mock-infected insects were injected with the same volume of complete TC-100. Injected larvae were observed daily for mortality, as determined by overall appearance and lack of response to agitation, until either death or pupation occurred. Larvae which died prematurely due to injection mortality were not considered in the final results.

Occluded virus yields. Larvae which were injected with the highest dose of each virus and which died from viral infection were collected at the time of death and stored at −20°C until the end of the experiment. Each group of larvae was pooled and homogenized in 1% SDS (1 ml per larva), and occlusion bodies were counted in a hemocytometer following dilution in distilled water. Two independent counts were made for each sample. The number of occlusion bodies per insect was taken as being equivalent to the number of occlusion bodies per milliliter.

RESULTS

Protein synthesis in cells infected with *p35* mutant viruses. The *p35* mutant viruses which were used in these experiments were described previously (3). Briefly, vAcAnh was isolated as a spontaneous mutant and was found to have a deletion affecting the *p35* gene and the adjacent *hr5* region, while vP35Z was constructed by replacement of a portion of *p35* with the *Escherichia coli lacZ* gene.

To characterize the effect of apoptosis on Ac*M*NPV infection at the cellular level, we examined protein synthesis profiles of these two different *p35* mutants in SF-21 cells (which undergo apoptosis) and in TN-368 cells (which do not undergo apoptosis). The pattern of protein synthesis in SF-21 cells infected with either vAcAnh or vP35Z differed substantially from that of cells infected with wt Ac*M*NPV (Fig. 1A). Although there was very little evidence of virally induced protein synthesis in cells infected with vAcAnh or vP35Z, two new proteins of M_r approximately 33,000 and 37,000 (33K and 37K proteins) were induced, but their synthesis appeared to be delayed relative to that of wt-infected cells. Both of these proteins are early proteins; their synthesis is observed in cells infected with wt Ac*M*NPV in the presence of aphidicolin (38), which blocks late gene expression by blocking DNA replication, and in cells infected with *ts*8, a conditionally lethal mutant blocked in DNA synthesis as a result of a mutation in a helicase-homologous gene (12, 25). In addition, the 33K polypeptide is clearly observed in SF-21 cells infected with the mutant *ts*B821, a temperature-sensitive mutant which is blocked in the early phase (29). There was no evidence of late (e.g., gp67 [44]) or very late (e.g., polyhedrin, 30K) protein synthesis in *p35* mutant-infected SF-21 cells (Fig. 1A). Host protein synthesis was not specifically shut off in *p35* mutant-infected SF-21 cells, as is normally the case for Ac*M*NPV infection. However, there was a general decline in protein synthesis between 18 and 24 h p.i., and by 48 h p.i., no protein synthesis was detected (Fig. 1A). In wt-infected cells, host protein synthesis began to decline approximately 18 to 24 h p.i., and virus-specific protein synthesis increased until, by 48 h p.i., polyhedrin synthesis predominated (Fig. 1A).

In contrast, the protein synthesis profiles of TN-368 cells

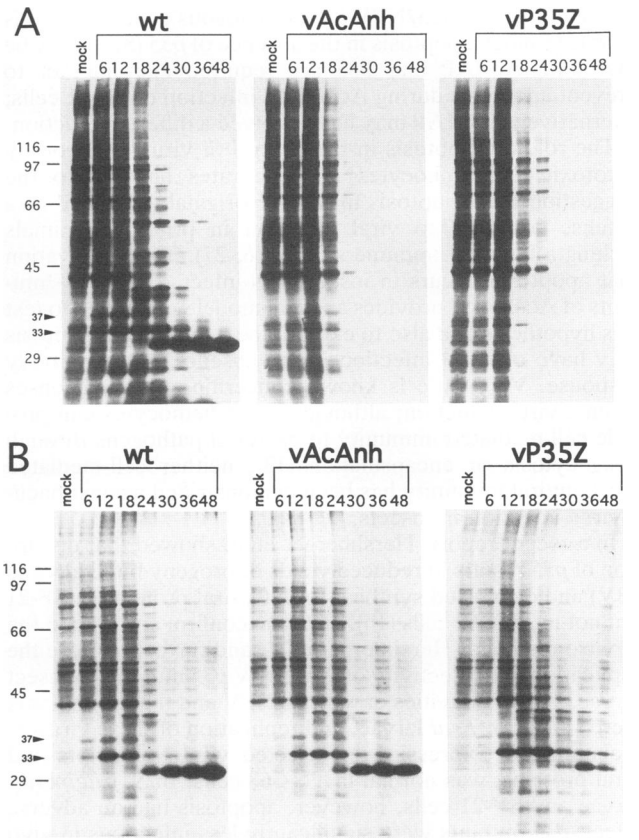

FIG. 1. Kinetics of protein synthesis in SF-21 (A) and TN-368 (B) cells infected with wt Ac*M*NPV, vAcAnh, or vP35Z. Cells were either mock infected or infected with the viruses shown, and proteins were pulse-labeled with [^{35}S]methionine for 1 h at the times indicated (hours p.i.). The positions of size markers are shown at the left in kilodaltons, and the positions of the 37K and 33K polypeptides discussed in the text are also indicated by arrowheads at the left.

infected with vAcAnh, vP35Z, or wt were similar in both the timing of appearance and the intensity of virally induced proteins as well as in the shutoff of host proteins (Fig. 1B). The only slight differences detected were a delay in the shutoff of the 33K virally induced early protein discussed above in *p35* mutant-infected lysates compared with wt-infected cells (compare the 24-h lanes) and low levels of polyhedrin in vP35Z-infected lysates compared with wt- and vAcAnh-infected lysates (compare the 24- through 48-h lanes). Both vP35Z and a revertant virus containing a wt *p35* gene (vP35ZRS; see below) were also somewhat defective in production of occlusion bodies in TN-368 cells (5), presumably because of an unidentified second-site mutation affecting polyhedrin synthesis. Comparison of mutant- and wt-infected proteins failed to reveal either a candidate P35 polypeptide or a P35–β-galactosidase fusion protein in the vP35Z-infected samples. Overall, these protein synthesis results are consistent with those reported by Hershberger et al. (16).

Analysis of steady-state levels of selected viral transcripts. In an effort to further characterize viral gene expression in SF-21 and TN-368 cells infected with *p35* mutant viruses, we examined the levels of transcripts from three viral genes in the three main transcriptional classes of Ac*M*NPV genes:

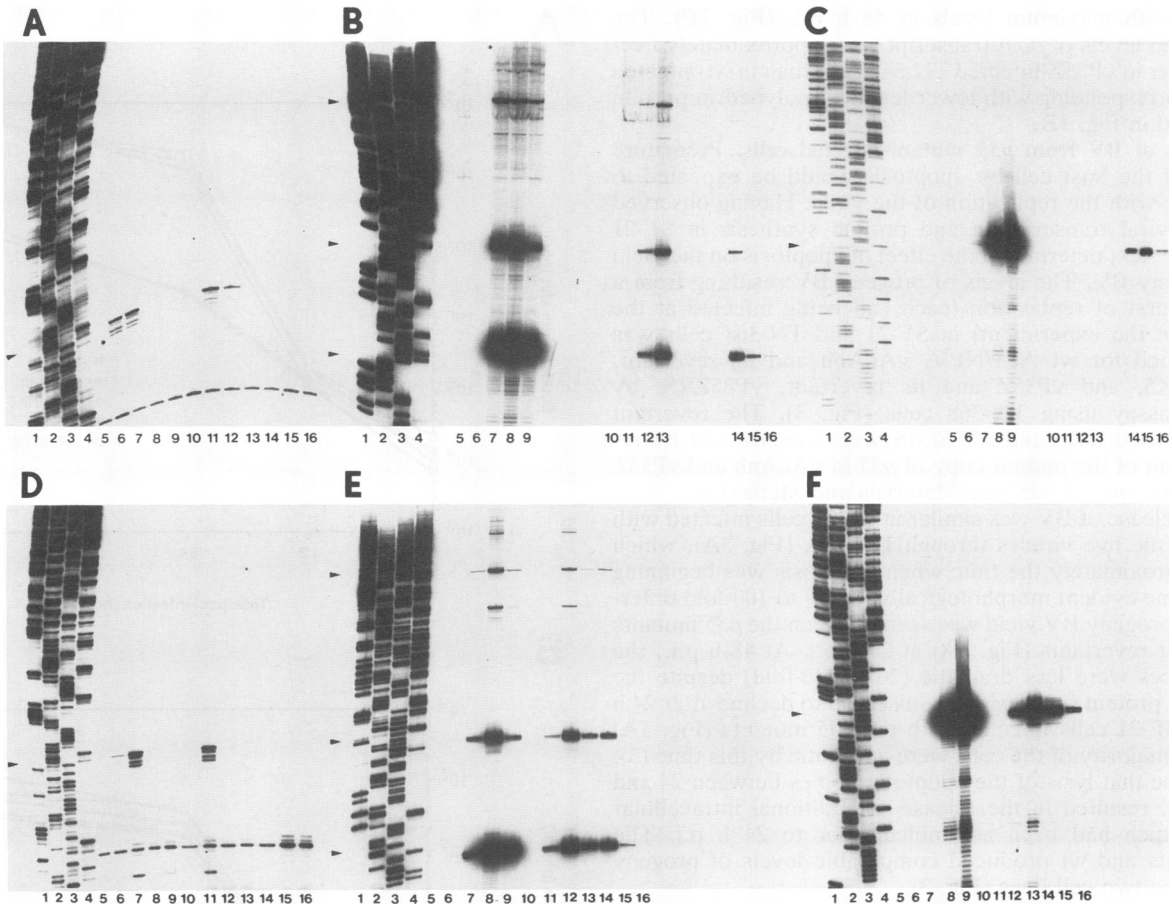

FIG. 2. Steady-state levels of selected viral transcripts during infection of SF-21 (A to C) and TN-368 (D to F) cells with wt AcMNPV or vP35Z. Total RNA was harvested from cells at various times p.i. and analyzed by primer extension, using oligonucleotides complementary to the early *egt* (A and D), the late *vp39* (B and E), or the very late *polh* (C and F) gene. Lanes: 1 to 4, G, A, T, and C lanes of dideoxynucleotide sequencing reactions using the same labeled oligonucleotide primers; 5, RNA from mock-infected cells; 6 to 9, RNA from 6-, 12-, 24-, and 48-h-p.i. cells infected with wt; 10 to 13, RNA from 6-, 12-, 24-, and 48-h-p.i. cells infected with vP35Z; 14 to 16, 1:5-, 1:25-, and 1:125-fold dilutions of the RNA in lane 6 (*egt*) or lane 8 (*vp39* and *polh*) (lanes 14 and 15 are reversed in panel C). Arrowheads at the left indicate the expected positions of the extension products for each oligonucleotide primer.

early, late, and very late. For this, we chose the AcMNPV genes *egt* (the gene encoding ecdysteroid UDP-glucosyltransferase [34]), *vp39* (the gene encoding the major capsid protein [39]), and *polh* (the gene encoding polyhedrin [18]), respectively. The level of transcripts from each of these three genes was examined by primer extension in both wt- and vP35Z-infected SF-21 (Fig. 2A to C) and TN-368 (Fig. 2D to F) cells. The results obtained with wt AcMNPV corresponded well with those previously published for these genes in both the temporal accumulation of the transcripts and location of the 5′ termini (32, 34, 39).

Transcripts from the early gene *egt* were delayed in their appearance and disappearance in vP35Z-infected SF-21 cells compared to wt-infected cells (Fig. 2A). Whereas the transcripts were detected at 6 and 12 h p.i. in wt-infected cells, they were not detected until 12 h p.i. in vP35Z-infected cells and were also present at 24 h p.i. This delay in accumulation of the *egt* transcript is consistent with the delay seen in the appearance of early proteins in SF-21 cells (Fig. 1A). Similar results were obtained (5) with use of an oligonucleotide primer specific for a different early viral gene, *pcna* (proliferating cell nuclear antigen) (7).

The accumulation of transcripts from the late *vp39* gene was also delayed in vP35Z-infected SF-21 cells (Fig. 2B). *vp39* transcripts were first detected in vP35Z-infected cells at 24 h p.i. and reached maximum levels at 48 h p.i., whereas in wt-infected cells, *vp39* transcripts were first detected at 12 h p.i. and reached maximum levels at 24 h p.i. (Fig. 2B). In addition, the maximum accumulation of *vp39* transcripts appeared to be approximately fivefold lower in vP35Z-infected SF-21 cells than in wt-infected cells.

Levels of transcripts from the very late *polh* gene were extremely low in vP35Z-infected SF-21 cells (Fig. 2C) and were barely detectable even upon overexposure of the autoradiograph (data not shown), whereas *polh* transcripts were first detected at 24 h p.i. in wt-infected cells and reached a maximum at 48 h p.i. (Fig. 2C).

In TN-368 cells (Fig. 2D to F), the levels of accumulation of *egt*, *vp39*, and *polh* transcripts were similar during infection with vP35Z or wt. Transcripts of *egt* were first detected at 6 h p.i., with maximum levels at 12 h p.i. in both vP35Z- and wt-infected TN-368 cells (Fig. 2D; a band corresponding to the *egt* transcript was clearly visible in lane 10 on the original autoradiograph), while *vp39* transcripts were detected at 12, 24 and 48 h p.i., with maximum levels at 24 h p.i. (Fig. 2E), and *polh* transcripts were detected at 24 and 48

h p.i., with maximum levels at 48 h p.i. (Fig. 2F). The maximum levels of *polh* transcript were approximately fivefold lower in vP35Z-infected TN-368 cells than in wt-infected cells, corresponding with lower levels of polyhedrin protein observed in Fig. 1B.

Yields of BV from *p35* mutant-infected cells. Premature death of the host cell by apoptosis would be expected to interfere with the replication of the virus. Having observed altered viral transcription and protein synthesis in SF-21 cells, we next determined the effect of apoptosis on the yield of progeny BV. The levels of progeny BV resulting from a single burst of replication (each cell being infected at the outset of the experiment) in SF-21 and TN-368 cells was determined for wt Ac*M*NPV, vAcAnh and its revertant, vAnhHK5, and vP35Z and its revertant, vP35ZRS, by plaque assay using TN-368 cells (Fig. 3). The revertant viruses vAnhHK5 and vP35ZRS were constructed by replacement of the mutant copy of *p35* in vAcAnh and vP35Z with a wt copy of *p35* (see Materials and Methods).

The release of BV was similar in SF-21 cells infected with each of the five viruses through 12 h p.i. (Fig. 3A), which was approximately the time when apoptosis was beginning to become evident morphologically. A 50- to 100-fold difference in progeny BV yield was seen between the *p35* mutants and their revertants (Fig. 3A) at 24 h p.i. At 48 h p.i., the differences were less dramatic (20- to 40-fold) despite the fact that protein synthesis was observed to decline after 24 h p.i. in SF-21 cells infected with the *p35* mutants (Fig. 1A) and the majority of the cells were apoptotic by this time (3). It may be that lysis of the apoptotic bodies between 24 and 48 h p.i. resulted in the release of additional intracellular virus which had been assembled prior to 24 h p.i. The revertants and wt produced comparable levels of progeny BV in the two cell lines (Fig. 3).

The levels of progeny BV obtained from infection of TN-368 cells with the five viruses were essentially identical at all time points examined (Fig. 3B). This result, in addition to the protein synthesis and RNA accumulation data presented above, indicates that *p35* is not required for normal replication in TN-368 cells.

These results are consistent with those reported by Hershberger et al., who examined BV yields at 48 h p.i. (16). Although they observed a much greater difference in levels of progeny BV obtained from SF-21 cells infected with a *p35* mutant and wt Ac*M*NPV (16), this larger difference was almost certainly due to the lower MOI that they used, which would allow for multiple rounds of replication and amplification of any existing differences between the mutants and wt.

Infectivity of *p35* mutants in insect larvae. Although the lack of the *p35* gene results in apoptosis and thereby a reduction in Ac*M*NPV replication in SF-21 cells, it was important to determine whether this effect also occurred in the whole organism or whether it was simply a cell line-specific effect. Since apoptosis reduces the yield of progeny BV from SF-21 cells, one prediction of the effect of an apoptotic response on virus infection in vivo would be an increase in the amount of virus required to initiate infection. We thus determined the approximate dosages of BV required for 50% lethality (LD_{50}s) of wt Ac*M*NPV, the two *p35* mutants vAcAnh and vP35Z, and their revertants vAnhHK5 and vP35ZRS, in *S. frugiperda* and *T. ni* larvae. As seen in Fig. 4A, the LD_{50} in fifth-instar *S. frugiperda* larvae was approximately 1,000-fold higher for the *p35* mutants than for wt Ac*M*NPV or their revertants. Wild-type Ac*M*NPV and both of the revertants had LD_{50}s between 10 and 100 PFU

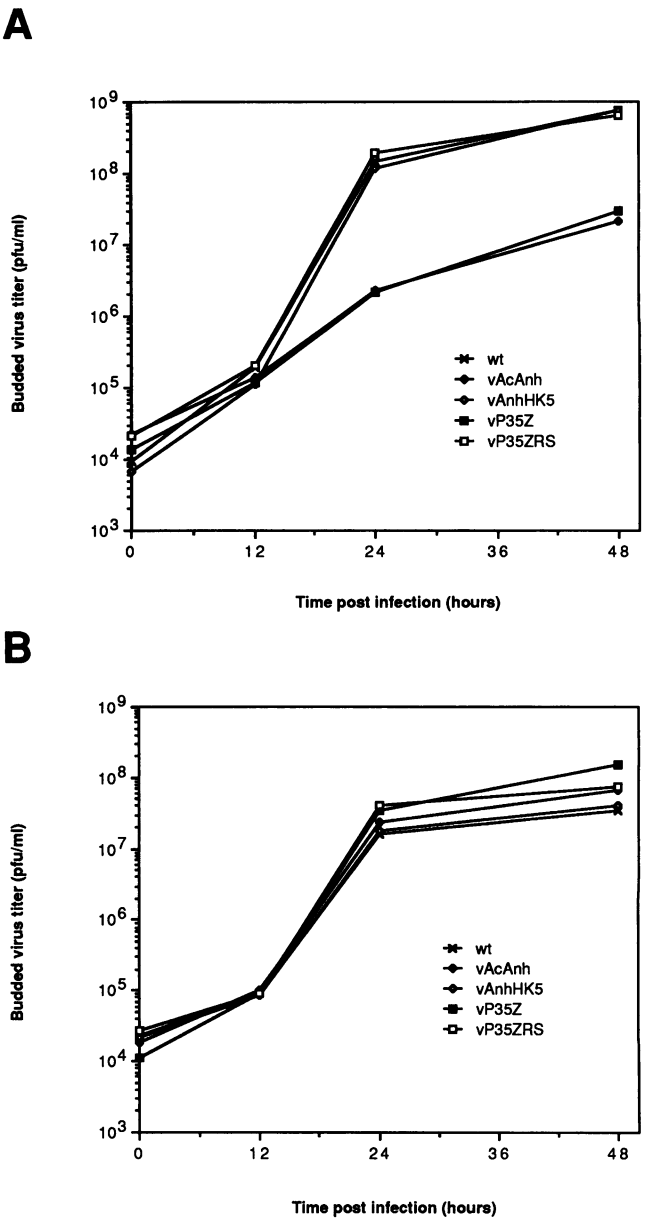

FIG. 3. Yield of progeny BV from SF-21 (A) and TN-368 (B) cells infected with wt Ac*M*NPV, the *p35* mutant vAcAnh or vP35Z, or the revertant virus vAnhHK5 or vP35ZRS. Cell monolayers were infected at an MOI of 20 PFU per cell, and samples of the culture supernatant were harvested at 0, 12, 24, or 48 h p.i. and titered by plaque assay using TN-368 cells. The results shown represent the averages of two independent experiments.

per larva, which were similar to previously reported results (9). Since the LD_{50}s of the revertants were similar to that of wt Ac*M*NPV, any differences seen between the two mutants and wt were due to the presence of the *p35* gene.

In fourth-instar *T. ni*, the LD_{50}s of all five of the viruses were between 1 and 10 PFU per larva (Fig. 4B). The lower LD_{50} of the budded form of Ac*M*NPV in *T. ni* compared with *S. frugiperda* has been reported previously (9). There were no significant differences among the LD_{50}s of wt, the *p35* mutants, or the revertant viruses in *T. ni* larvae. Thus, the

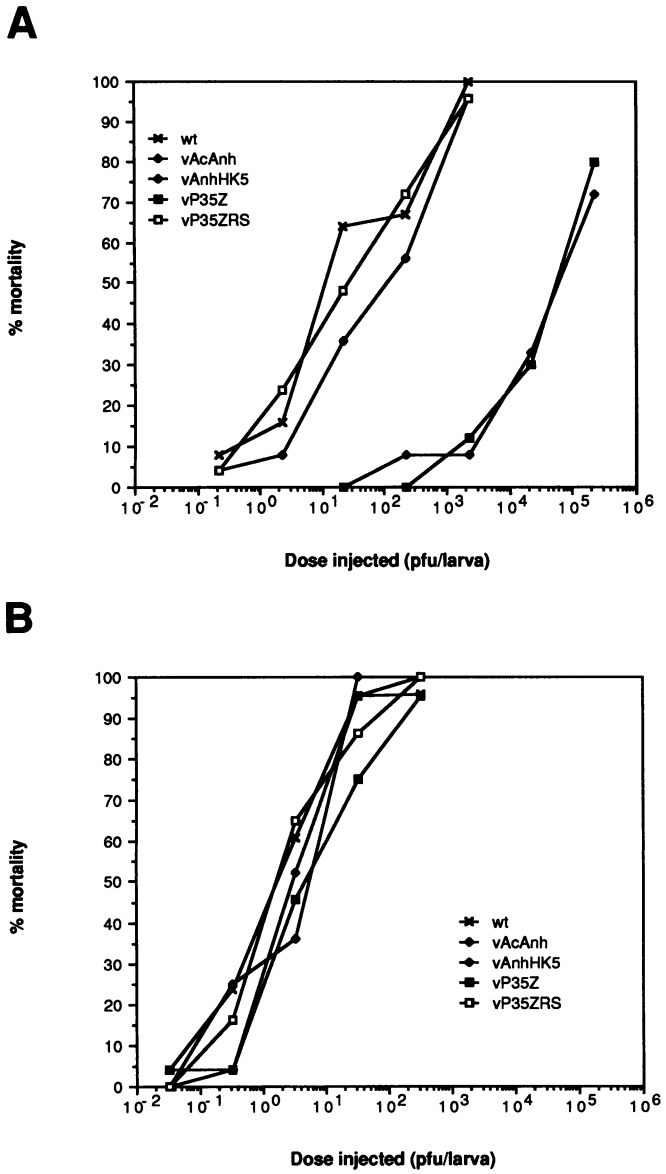

FIG. 4. Mortality due to virus infection in *S. frugiperda* (A) and *T. ni* (B) larvae injected with various doses of wt Ac*M*NPV, vP35Z, vAcAnh, vAnhHK5, or vP35ZRS. Twenty-five larvae were injected per dose; percent mortality was calculated as the number of dead larvae (minus larvae which died as a result of the injection procedure) divided by the number of larvae which survived the injection procedure (at least 20, except for the highest doses of wt and vAcAnh in *T. ni*, which were 19 and 18, respectively). No mortality was observed in mock-infected larvae.

differential response of SF-21 and TN-368 cells to infection with the *p35* mutants was also observed at the organismal level.

Another prediction arising from an apoptotic response in larvae would be a large reduction in the yield of the occluded form of the virus (OV), since *p35* mutant-infected SF-21 cells do not produce any occlusion bodies (3). We therefore determined the yield of OV from the larvae which died from viral infection at the highest doses injected. *S. frugiperda* larvae infected with vAcAnh produced approximately 900-fold less OV than did larvae infected with the revertant virus vAnhHK5, whereas approximately 4-fold less OV was produced in *T. ni* larvae infected with vAcAnh than in larvae infected with vAnhHK5 (Table 1).

TABLE 1. OV yields from *S. frugiperda* and *T. ni* larvae[a] infected with wt Ac*M*NPV, vAcAnh, or vAnhHK5

Host	Virus	OV/larva[b] ± SE[c]
S. frugiperda	wt	$6.5 \times 10^8 \pm 6 \times 10^7$
	vAcAnh	$1.4 \times 10^6 \pm 1 \times 10^5$
	vAnhHK5	$1.2 \times 10^9 \pm 2 \times 10^7$
T. ni	wt	$1.8 \times 10^8 \pm 2 \times 10^7$
	vAcAnh	$2.7 \times 10^7 \pm 9 \times 10^5$
	vAnhHK5	$1.0 \times 10^8 \pm 3 \times 10^6$

[a] The larvae used were those which were injected with the highest doses of BV in Fig. 4 and which died from virus infection.
[b] Larvae were pooled and homogenized in a total volume of buffer equivalent to the number of larvae per sample (in milliliters).
[c] Based on two independent counts of the same sample.

A significant difference was also seen in the melting and liquefaction of the cadavers between the larvae injected with the two *p35* mutants versus wt or the two revertants. Late in wt Ac*M*NPV infection, the infected cadaver normally breaks down as the muscles and other tissues liquefy, and the contents of the dead larva (mainly OV) are released into the environment. Both *S. frugiperda* and *T. ni* larvae infected with vP35Z or vAcAnh did not melt; although the insects became flaccid, the cuticle did not rupture. Many of the dead larvae infected with the *p35* mutants also did not melanize. Melting was normal in larvae infected with wt or the revertant viruses.

DISCUSSION

Although the requirement for the Ac*M*NPV *p35* gene product in blocking programmed cell death in the SF-21 cell line has been demonstrated previously (3, 16), it was not known whether *p35* was required only in cell culture or whether it also plays a similar role in the natural host. Our results show that mutation of *p35* results in greatly reduced levels of Ac*M*NPV replication and infectivity in both SF-21 cells and *S. frugiperda* larvae but not in TN-368 cells or *T. ni* larvae.

T. ni and *S. frugiperda* larvae appear to have little or no effective defense against infection by wt Ac*M*NPV via injection of BV into the hemocoel. Very few infectious virus particles (PFU determined by plaque assay in tissue culture) were required to establish a lethal infection in larvae of these two species, with the LD_{50} for wt Ac*M*NPV being between approximately 1 and 10 PFU per larva in *T. ni* and between 10 and 100 PFU per larva in *S. frugiperda*. The higher LD_{50} in *S. frugiperda* was probably due not to an organismal defense system but to the intrinsically lower infectivity of Ac*M*NPV in *S. frugiperda* cells; titers of wt Ac*M*NPV determined in *S. frugiperda* cell lines are consistently 5- to 10-fold lower than titers determined in *T. ni* cell lines (5, 9, 26). Thus, any host defense to virus invasion which may exist in these insects appears to be inadequate in preventing lethal infection by wt Ac*M*NPV.

Inactivation of the Ac*M*NPV *p35* gene resulted in an increase in LD_{50} of 3 orders of magnitude in *S. frugiperda* larvae. Since infection with *p35* mutants results in apoptosis in SF-21 cells, the most straightforward interpretation of this result is that an apoptotic response by cells in the *S. frugiperda* larvae decreased the ability of the *p35* mutant viruses to establish a lethal infection. The observation that

the LD_{50}s of the *p35* mutants were equivalent to that of wt in *T. ni* larvae served as a control, since the *p35* mutants did not cause apoptosis in TN-368 cells. At the current time, however, we do not have supporting data to demonstrate that apoptosis is occurring in vivo. Apoptotic cells were not consistently observed in the hemolymph of *p35* mutant-infected insects (5), although this may have been due to a combination of the asynchronous nature of in vivo infections and rapid phagocytosis of apoptotic cells by hemocytes.

Regardless of the mechanism by which *p35* functions to increase infectivity in *S. frugiperda*, its presence would be expected to provide a clear evolutionary advantage for Ac*M*NPV replication in this species. *p35* therefore effectively constitutes a host range determinant in Ac*M*NPV, and acquiring *p35* has probably allowed the virus to expand its practical host range to other species, including *S. frugiperda*.

Although the LD_{50} for the *p35* mutants was approximately 1,000-fold higher than that of wt in *S. frugiperda* larvae, the decrease in BV yield from SF-21 cells infected with *p35* mutants was only 50- to 100-fold compared with the wt level. This difference might be explained by several factors, including the following: (i) the high MOI used to infect SF-21 cells may have accelerated the course of infection and increased the amount of BV produced before apoptosis was fully implemented, and (ii) during in vivo infection, phagocytic cells may recognize apoptotic cells and engulf them before large amounts of BV are released, resulting in inactivation of intracellular virus (6).

The differential response to *p35* mutant infection in *S. frugiperda* and *T. ni* appears to be largely species specific; species-specific effects were observed in both in vivo infectivity and OV production. However, the lack of melting in both *S. frugiperda* and *T. ni* larvae infected with *p35* mutants indicates that infection of both species is affected to some degree by inactivation of *p35*. The physiological basis for larval melting is unknown, and the connection between melting and apoptosis, if one exists, is far from clear at this time. Since a normal melting phenotype was restored in the *p35* revertants vAnhHK5 and vP35ZRS, it is unlikely that a second-site mutation was responsible for the lack of melting. It is possible that melting requires the infection of a specific tissue of the larva or that it requires the production of a melting factor. If P35 is required for either of these possibilities, then melting would not occur in *p35* mutant-infected larvae of either species.

It is possible that the species-specific effects of *p35* mutation lie in a difference in the proportions of types of cells or tissues that are sensitive to apoptosis in the two species. This explanation is supported by the observation that a low level of OV was produced in *S. frugiperda* larvae infected with vAcAnh, indicating that some tissues of this species allowed completion of the viral replication cycle and consequent production of OV. In addition, three- to fourfold less OV was produced in *T. ni* larvae infected with vAcAnh than in larvae infected with vAnhHK5, suggesting that there may be a small proportion of tissues which undergo apoptosis in this species as well. Although we have not extensively studied the effect of larva-to-larva variability on OV yield, initial experiments on individual *S. frugiperda* larvae infected with *p35* mutant viruses indicated that a low level of OV was produced in each larva (5).

Our results contrast with those of Kamita et al. (21), who studied the in vivo effect of mutating the BmNPV *p35* gene by injecting *B. mori* larvae with BV. No differences were observed between wt BmNPV and the *p35* mutant in terms of infectivity, OV yield, or survival time. However, these results are difficult to interpret given the mixed in vitro phenotype of their mutant virus (21). Furthermore, only very high dosages of BV (5×10^5 PFU per larva) were used (21), so that any difference in LD_{50} between their *p35* mutant and wt BmNPV may not have been detectable.

Analysis of gene expression in SF-21 cells infected with *p35* mutants revealed a delay in early gene expression, a failure of the mutant to shut off host protein synthesis in a timely fashion, a defect in the ability of the mutants to initiate late protein synthesis, and an eventual decline in total protein synthesis in apoptotic cells. The pattern of protein synthesis in these cells indicated that the only viral proteins synthesized were primarily those of the early class and that their appearance was delayed compared with wt infection. Similar protein synthesis patterns were obtained by Hershberger et al. (16), and a delay in the synthesis of several early viral proteins is also apparent in their data. Analysis of the transcription of selected viral genes revealed a delay in both the early (*egt*) and late (*vp39*) transcriptional phases and a significant reduction in the levels of late and very late (*polh*) gene transcription. The virtual lack of late protein synthesis despite the presence of some level of late RNAs may be related to the observation that total protein synthesis is shut off by 36 h p.i., a time at which most cells are apoptotic. If apoptosis is accompanied by a shutoff in protein synthesis, then cells which initiate late gene transcription might synthesize some late viral RNAs but little or no synthesis of late proteins would be observed. Similar results have been reported with a herpes simplex virus type 1 mutant which induces programmed cell death in neuroblastoma cells (2). It remains to be determined whether the shutoff of protein synthesis is a general phenomenon of apoptotic cells or a characteristic only of virally infected apoptotic cells.

The data show that P35 accelerates Ac*M*NPV infection in SF-21 cells, and we currently favor the hypothesis that P35 accelerates and/or intensifies, directly or indirectly, the expression of other viral genes, including at least one which is directly responsible for blocking apoptosis. The delay or reduction in the level of early gene expression could provide enough time for SF-21 cells to mount an irreversible apoptotic defense to *p35* mutant infection. In contrast, *p35* expression in wt-infected cells would allow timely and sufficient expression of early and late genes so that the apoptotic program, even if transiently initiated, can be blocked. Hershberger et al. (16) have suggested that P35 may be involved in the general infectivity of Ac*M*NPV in SF-21 cells; this would also be consistent with a role for P35 in accelerating the infection process, particularly if timely and effective expression of early viral genes is critical to the viral invasion strategy. We suspect that the observed delay in early viral gene expression is a particularly critical feature of the *p35* mutant phenotype because cells infected with a mutant of Ac*M*NPV carrying a null mutation in the viral *pcna*-homologous gene show a delay specifically in DNA replication and the initiation of late gene expression, but these cells do not undergo apoptosis (7, 33).

Further, we prefer the view that P35 or the factor which it regulates acts directly in blocking apoptosis in SF-21 cells because apoptosis appears to be initiated at the same time in both wt- and *p35* mutant-infected SF-21 cells; cell surface blebbing, a morphological change evident during the early stages of apoptosis, is observed at the same time (approximately 12 h p.i.) in both cases (3). In wt-infected cells, this blebbing is transient and subsides during the later stages of

infection, whereas blebbing intensifies as *p35* mutant infections proceed. If apoptosis is indeed initiated in both wt- and *p35* mutant-infected SF-21 cells, then P35 or the antiapoptotic viral gene product(s) stimulated by P35 function is likely to act by directly blocking the apoptotic program at some crucial point.

The hypothesis that timely expression of at least one early viral gene product can block apoptosis in *S. frugiperda* cells is strongly supported by our previous observation that wt AcMNPV, but not vAcAnh, can prevent apoptosis triggered by actinomycin D treatment if the actinomycin D is added at 5 h p.i. or later, but wt infection cannot block apoptosis if the actinomycin D is added prior to 5 hours p.i. (8). These experiments do not distinguish whether P35 acts directly or activates the expression of other viral gene products to block actinomycin D-induced apoptosis.

The fact that the CpGV *iap* gene, a zinc finger-like gene which is distinct from *p35*, can functionally substitute for *p35* in blocking *p35* mutant-induced or actinomycin D-induced apoptosis in SF-21 cells (8) provides additional perspective to the question of P35 function. Since the CpGV *iap* gene has a homolog (Ac-*iap*) in the AcMNPV genome and Ac-*iap* is unable to functionally substitute for the AcMNPV *p35* gene in blocking apoptosis (8), it is possible that P35 regulates Ac-IAP activity in some way, either by direct interaction with Ac-IAP at the protein level or by stimulation of Ac-*iap* expression. In either case, a central (but not necessarily direct) role for *iap* genes in blocking apoptosis in SF-21 cells is indicated. Since the predicted products of these genes have zinc finger-like motifs, they are likely to act at the level of gene regulation, but whether they act directly to regulate the cellular genes involved in implementing the apoptosis program or whether they act to regulate other viral genes remains to be determined.

The observation that viral protein synthesis and transcription were normal in *p35* mutant-infected TN-368 cells leads to the question of what specific role P35 plays in SF-21 cells or, alternately, why P35 is not required in TN-368 cells. There are at least four possibilities why P35 function may be unnecessary for normal replication in *T. ni* cells. (i) AcMNPV may not trigger apoptosis in this species so that P35 or P35-regulated factors which are required to block the programmed response are not necessary. Support for this view comes from the observation that the cell surface blebbing indicative of early apoptosis is not observed even transiently in TN-368 infections (3). However, apoptosis is a common response to infection by large DNA-containing viruses (4) and appears to be an important organismal defense to viral invasion, so it would be unclear why *T. ni* cells lack this response. (ii) There may be a second (redundant) pathway by which AcMNPV is able to block apoptosis in *T. ni* cells but which is ineffective in *S. frugiperda* cells. If this pathway worked so as to block even the initiation of apoptosis, then neither initial cell surface blebbing nor eventual apoptosis would be observed in *T. ni* cells. However, this would not explain why the presence of intact *p35* accelerates infection in SF-21 cells but not TN-368 cells. (iii) The regulation of early gene transcription in *T. ni* and *S. frugiperda* cells may differ so significantly that P35 may be required for timely and intense early gene transcription only in *S. frugiperda* cells. Support for this view comes from the observation that the regulation of transcription of at least one early AcMNPV gene differs substantially in TN-368 and SF-21 cells; the primary 1.7-kb transcript of the DA26 ORF of AcMNPV is synthesized earlier in TN-368 cells than in SF-21 cells (36). (iv) The *p35*-regulated viral gene product(s) which blocks apoptosis may interact more effectively in TN-368 cells than in SF-21 cells and is therefore required in less abundance to be equally effective in blocking apoptosis. These four possibilities may not be mutually exclusive, and one or some combination of them may be involved.

We conclude that cellular apoptosis can have a drastic effect on the outcome of AcMNPV infection in the insect host. Our results indicate that apoptosis can play a major role in insect immunity to virus infection and that the ability to block apoptosis is a determining factor in baculovirus host range. Our data also show that P35 accelerates the infection of SF-21 cells and suggest a role for P35 in accelerating or intensifying the synthesis of early viral gene products. One or more of these viral gene products may be directly responsible for blocking the cellular apoptosis program.

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