Expression of the *Autographa californica* Nuclear Polyhedrosis Virus Apoptotic Suppressor Gene *p35* in Nonpermissive *Spodoptera littoralis* Cells†

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Apoptosis was postulated as the main barrier to replication of the *Autographa californica* nuclear polyhedrosis virus (*AcMNPV*) in a *Spodoptera littoralis* SL2 cell line (N. Chejanovsky and E. Gershburg, Virology 209:519–525, 1995). Thus, we hypothesized that the viral apoptotic suppressor gene *p35* is either poorly expressed or nonfunctional in *AcMNPV*-infected SL2 cells. These questions were addressed by first determining the steady-state levels of the *p35* product, P35, in *AcMNPV*-infected SL2 cells. Indeed, very low levels of P35 were found in infected SL2 cells in comparison with those in SF9 cells. Overexpression of *p35*, in transient-transfection and recombinant-virus infection experiments, inhibited actinomycin D- and *AcMNPV*-induced apoptosis, as determined by reduced cell blebbing and release of oligonucleosomes and increased cell viability of SL2. However, SL2 budded-virus (BV) titers of a recombinant *AcMNPV* which highly expressed *p35* did not improve significantly. Also, injection of *S. littoralis* larvae with recombinant and wild-type *AcMNPV* BSs showed similar 50% lethal doses. These data suggest that apoptosis is not the only impediment to *AcMNPV* replication in these nonpermissive *S. littoralis* cells, and probably in *S. littoralis* larvae, so *p35* may not be the only host range determinant in this system.

The *Autographa californica* nuclear polyhedrosis virus (*AcMNPV*) is considered the prototype of subgroup A of the *Baculoviridae* family of viruses which infect invertebrates and primarily insects. The complete genome of *AcMNPV* has been sequenced (1), and its replication in permissive cells has been extensively studied (for reviews, see references 4 and 23). The ability of *AcMNPV* to infect a wide number of hosts, 39 species of lepidopteran larvae belonging to 13 families (5, 17), and the availability of appropriate tissue culture systems have facilitated the isolation of genetically engineered *AcMNPVs* with novel insecticidal properties. Some of these are being evaluated for agricultural application (reviewed in references 6 and 30).

The mechanisms that control the ability of baculoviruses to infect specific insect hosts are not clear. Their elucidation is important for better assessment of potential risks associated with the utilization of recombinant-baculovirus insecticides. Moreover, this knowledge could help expand the range of applications of specific *AcMNPV* recombinants to target economically important lepidopteran pests. Four baculovirus genes have been implicated in facilitating *AcMNPV* replication in a species-specific manner. (i) *p35*, characterized as an antiapoptotic gene, inhibitor of cysteine proteases of the CED-3/ICE family, caspases (2, 7, 24, 39), is required for efficient *AcMNPV* infection of *Spodoptera frugiperda* cells and larvae. *AcMNPV* *p35* gene null mutants yielded low titers of budded virus (BV) (12, 21). Moreover, the absence of *p35* function was complemented by apoptosis inhibitor genes (iap genes) derived from other baculoviruses (3, 15). (ii) A recombinant *AcMNPV* bearing a small fragment of the helicase gene of *Bombyx mori* nuclear polyhedrosisvirus (NPV) within *AcMNPV*’s *p143* coding region (the helicase gene of *AcMNPV*) replicated in both permissive *S. frugiperda* SF21 and nonpermissive *B. mori* BmN4 cell lines (14, 28). (iii) The *hrf-1* gene of the *Lymnaea dispar* NPV was shown to allow the multiplication of *AcMNPV* in nonpermissive *Ld652Y* cells (16, 37). (iv) The *lef-1* gene of *AcMNPV* was necessary for successful infection of TN368 cells and to some extent for improvement of the infectivity of the virus in *Trichoplusia ni* larvae but was not necessary for replication or infectivity in *S. frugiperda* cells and larvae, respectively (27).

In addition, 18 baculovirus genes (*lef* genes) were required for expression of a late baculovirus promoter in SF21 cells (26, 38); three of them, *ie-2, lef-7*, and *p35*, were not required for expression in TN368 cells, suggesting that they could be involved in determining host range.

*AcMNPV* does not infect *Spodoptera littoralis* (the Egyptian cotton worm), an important Mediterranean pest. Recently, we reported that infection of *S. littoralis* SL2 cells with wild-type *AcMNPV* results in apoptosis and concomitantly low yields of viral progeny (9). Since *AcMNPV* mutants with alterations in the *p35* gene induce apoptosis of the permissive cell line of *S. frugiperda*, SF21, we hypothesized that *p35* either is expressed poorly or is not functional, or both, in *AcMNPV*-infected SL2 cells (9). *p35* has been shown to suppress apoptosis in various heterologous systems (18, 20, 29, 33, 35) which are evolutionarily more distant from SF9 cells than the SL2 system (which is closely related to the *S. frugiperda* lepidopterous cells). This led us to hypothesize that *p35* may be functional but not sufficiently expressed in *AcMNPV*-infected SL2 cells. Thus, augmenting the expression of *p35* in SL2 cells could allow us to determine its functionality in terms of suppression of apoptosis. In the present work, we show that by overexpressing *p35* we were indeed able to reduce apoptosis of SL2 cells induced by either actinomycin D or *AcMNPV*. However, increasing *p35*...
expression only slightly improved the yields of AcMNPV BV in infected SL2 cells and did not improve the 50% lethal doses (LD_{50}, 8) of AcMNPV in *S. littoralis* fourth-instar larvae.

**RESULTS**

**P35 synthesis in AcMNPV-infected SL2 cells.** To compare the steady-state levels of P35 synthesized during the infectious cycle, both nonpermissive SL2 and permissive SF9 cells were infected with AcMNPV. At various times postinfection (p.i.), cell extracts were subjected to immunoblot analysis using α-P35NF antiserum (Fig. 1). P35 was detected in virus-infected SF9 cells at 4 h p.i. and reached its maximal expression at about 18 to 34 h p.i. (Fig. 1B). In SL2 cells, very small amounts of P35 were observed at 4 h p.i.; maximal steady-state levels were detected at 18 h p.i. (Fig. 1A). However, P35 steady-state levels in SL2 cells dropped from 18 to 34 h p.i., probably due to cell death (compare Fig. 1A and B). Overall, P35 levels in those cells were extremely low compared to those in infected SF9 cells.

These results suggested that a lack of sufficient amounts of P35 allows the progression of apoptosis initiated by AcMNPV infection of SL2 cells (9). However, this hypothesis did not rule out the possibility of p35's nonfunctionality in SL2 cells.

**Transient expression of p35 in SL2 cells.** We assumed that increasing the amount of P35 was a prerequisite to addressing the question of whether p35 is functional in SL2 cells. Previous studies have shown that high P35 levels are obtained in SF21 cells by expressing p35 under the control of the *hsp70* heat shock promoter from *Drosophila* (13). We compared the levels of P35 obtained during transfection of two plasmids, pBB/BSst and pHSP35VI+, in which p35 was placed under the control of its own promoter or the *hsp70* promoter, respectively (see Materials and Methods). A third plasmid, pIE1 (19), bearing the *ie-1* gene coding for IE1, a transactivator of the *ie-1* promoter, was transfected into SF9 cells, which was suppressed by cotransfection with pBB/BSst. Immunoblot analysis detected higher levels of P35 in the pHSP35VI+-transfected cells than in cells transfected with pBB/BSst plus pIE1, and the levels were undetectable in pBB/BSst-transfected cells (Fig. 2A). On the basis of these results, we selected the pHSP35VI+ construct for our expression studies (see below).

**p35-mediated suppression of apoptosis.** Actinomycin D treatment has been shown to induce apoptosis of lepidopteran SF21 cells, which was suppressed by p35 expression (8, 13). SL2 cells undergo apoptosis upon incubation with actinomycin D at concentrations above 100 ng/ml. The ability of p35 to block apoptosis in actinomycin D-
treated SL2 cells was studied by transfecting them with pHSP35VI and further incubating them with 250 ng of actinomycin D per ml. The time course of the apoptosis provoked by the addition of actinomycin D was monitored by using a semiquantitative ELISA-based assay which measures the amount of oligonucleosomal DNA released by the apoptotic cells (see Materials and Methods). A decrease of about 50% in the release of oligonucleosomal DNA was observed in SL2 cells transfected with pHSP35VI relative to that in control pBluescript-transfected cells (Fig. 3A). Untreated cells (no addition of actinomycin D) did not release oligonucleosomal DNA, and their optical densities were below 0.05. As expected, P35 was detected in the pHSP35VI-transfected cells and was not detected in the pBluescript-transfected cells (Fig. 3B, lanes 2 to 5 and 6, respectively). pBB/BSst-transfected cells released amounts of oligonucleosomes equivalent to those released by pBluescript-transfected cells, and P35 was barely detectable (data not shown and Fig. 2A).

A total of 60% of pHSP35VI-transfected SL2 cells remained viable after exposure to actinomycin D, in contrast to 18% of pBluescript (or pBB/BSst)-transfected cells (Fig. 4 and data not shown), again indicating that high-level expression of p35 conferred resistance to apoptosis.

**p35 overexpression during viral infection.** The experiments described above indicated that p35 expression in SL2 cells (i) could be elevated by placing its transcription under the control of the hsp70 promoter and (ii) resulted in a functional product, since it correlated with acquired resistance to actinomycin D-induced apoptosis. Thus, to study the ability of P35 to block AcMNPV-induced apoptosis, we constructed a recombinant virus (vHSP-P35) in which the hsp70 promoter-p35 transcription unit was inserted at the polyhedrin locus of the AcMNPV genome (Fig. 5).

SL2 cells were infected with vHSP-P35 at multiplicities of infection (MOIs) of 1 and 10, and p35 expression was detected as described above. Higher steady-state levels of P35 were produced in cells infected with the recombinant than in cells infected with wt AcMNPV (Fig. 6, lanes 2, 4, and 5 and lanes 3 and 6, respectively). Moreover, heat shocking the cells after infection resulted in a dramatic increase in P35 synthesis in the recombinant-virus-infected cells (Fig. 6). Apoptosis of the SL2 cells infected with wt and recombinant vHSP-P35 viruses (MOI of 10) is shown in Fig. 7. Electrophoresis of DNA extracted from AcMNPV-infected cells shows the typical oligonucleosomal ladder characteristic of apoptosis (Fig. 7A, lane 1), corresponding to the extensive cell death observed (Fig. 7C). Partial suppression of apoptosis was consistently observed in vHSP-P35-infected cells, as evidenced by the reduced extent of DNA fragmentation (Fig. 7A, lane 2 and 7B).
We estimated a reduction of about 50% apoptosis in vHSP-P35-versus wt-infected SL2 cells by densitometric scanning of the gel and, independently, by counting intact versus blebbing vHSP-P35- and wt-infected SL2 cells (Fig. 7).

**Inhibition of apoptosis and viral yields.** Apoptosis has been implicated in the significant reduction of BV yields observed in SL2 cells relative to virus yields measured in SF9 cells (9). Since overexpression of p35 reduced the extent of virus-induced apoptosis of SL2 cells, we determined the amount of progeny BVs released through one cycle of replication by infecting the cells with wt (AcMNPV) and recombinant (vHSP-P35) viruses at an MOI of 10 PFU per cell. As can be seen in Fig. 8, vHSP-P35 yields increased slightly compared to those of AcMNPV (vHSP-P35 titers of 5.17 × 10^6 ± 3.31 × 10^5 and 1.01 × 10^7 ± 8.4 × 10^5 and AcMNPV titers of 1.55 × 10^7 ± 1.29 × 10^6 and 2.48 × 10^7 ± 2.06 × 10^6 PFU/ml at 24 and 48 h p.i., respectively). Also, a slight increase in BV yields was noted with cells infected at a MOI of 1 (not shown). vHSP-P35 titers from permissive SF9 cells infected at 48 h p.i. (same MOI as above) were 1.01 × 10^7 ± 8.3 × 10^6 and 2.00 × 10^8 ± 1.1 × 10^7 PFU/ml, with or without the cells being heat shocked, respectively. AcMNPV titers under the same conditions were 3.05 × 10^7 ± 2.40 × 10^6 and 6.10 × 10^8 ± 3.50 × 10^8 PFU/ml, with or without the cells being heat shocked, respectively.

Routine determination of BV released from S. littoralis NPV (SNPV)-infected SL2 cells yielded titers of about 10^9 PFU/ml (9).

In order to determine if p35 overexpression improved the ability of AcMNPV to replicate at the organism level, we determined the approximate LD_{50} of vHSP-P35 and wt AcMNPV for fourth-instar S. littoralis larvae. That was accomplished by infecting vHSP-P35 and AcMNPV BV doses ranging from 10^2 to 10^6 PFU per larva. As can be seen in Fig. 9, approximate LD_{50} of 10^3 PFU/larvae were determined for vHSP-P35 and wt AcMNPV, by graphic extrapolation. Thus, no significant differences in LD_{50} between recombinant and wt AcMNPVs in nonpermissive S. littoralis larvae were observed.

**DISCUSSION**

Virus-induced apoptosis fully develops in SL2 and is suppressed in SF21 (or SF9) cells infected with wt AcMNPV (7, 9, 11, 22). To determine whether AcMNPV’s failure to suppress apoptosis in infected SL2 cells is due to poor expression of the apoptotic suppressor gene p35, we compared the steady-state levels of P35 in SL2 and SF9 cells infected with AcMNPV. P35 levels were indeed much lower in infected SL2 cells than in their SF9 counterparts (Fig. 1A and B, respectively). Studies of P35’s mode of action have indicated that it binds stoichiometrically to CED-3/ICE-like death proteases of invertebrate and vertebrate origin, inhibiting their activity (2, 7, 24, 39). Thus, the above results suggest that apoptosis in infected SL2 cells could occur due to insufficient amounts of P35 available to bind to and inhibit a putative SL2 CED-3/ICE-related protease activated by AcMNPV. Alternatively, P35 levels in nonpermissive SL2 cells could be equivalent to those in permissive cells, but p35 could be nonfunctional, unable to suppress apoptosis, i.e., P35 could either not bind or bind with lower affinity to the SL2 caspase (2). A prerequisite to examination of these alternatives was overexpression of p35.

Expression of p35 directed by the Drosophila hsp70 promoter resulted in remarkably higher steady-state levels of P35 in both plasmid-transfected and recombinant-virus-infected
SL2 cells (Fig. 2 and 6). p35 expression was further enhanced by heat shocking the cells (Fig. 6 and data not shown).

**Does p35 overexpression protect SL2 cells from apoptosis?** Apoptosis of SL2 cells was induced by actinomycin D and AcMNPV infection. In the case of actinomycin D-treated cells, p35 expression correlated with inhibition of apoptosis, estimated directly by measuring the amount of oligonucleosomal DNA released by the cells (Fig. 3), and as evidenced by higher cell viability (Fig. 4). Overexpression of p35 has been shown to augment viability of SF21 cells, protecting them from apoptosis induced by either actinomycin D or expression of the p32 form of the human ICE protease gene (7, 13).

Recombinant vHSP-P35 infection also induced apoptosis of SL2 cells, albeit to a lower extent than wt AcMNPV (Fig. 7A).

FIG. 7. Overexpression of p35 reduces apoptosis of SL2 cells. (A) Intracellular DNA fragmentation. DNA was extracted from SL2 cells infected with wt virus or vHSP-P35 virus (lanes 1 and 2, respectively). Lane M, λ DNA digested with BstEII. (B and C) SL2 cells infected with vHSP-P35 and the wt, respectively. Light micrographs (magnification ×200) were prepared as previously described (9). Intact and blebbing cells were counted (250 to 350 cells per field, three replicates). The extent of apoptosis of vHSP-P35-infected SL2 cells relative to that of wt-infected cells was estimated.
The infected cells needed to be heat shocked in order to obtain significant p35 expression and exhibit reduced cellular DNA fragmentation, blebbing, and cell destruction (Fig. 6 and 7). Apoptosis of SF21 cells induced by a p35 null mutant virus, vAcAnh, was prevented by p35 expression, and this correlated with the ability of p35 to prevent actinomycin D-induced apoptosis of SF21 cells (13). Another study has shown that SF21 cells stably transformed to express p35 are resistant to apoptotic death induced by actinomycin D (8). Taken together, these and our results lead to the conclusion that a functional P35, product of p35 expression, protects the SL2 cell from apoptotic death induced by actinomycin D or AcMNPV infection.

Our inability to achieve 100% suppression of apoptosis by overexpressing p35 could be due to the lack of synchronization of the infected SL2 cells. Thus, different cells at different phases of the cell cycle may possess differential sensitivities to induction, and suppression, of apoptosis (24). One way to overcome this problem could be the isolation of SL2 cells stably transformed to express p35 (reference 8 and see below).

P35 levels and AcMNPV progeny yields. Apoptosis, conceived as an antiviral response of the host, blocks viral replication and reduces virus yields (8, 9, 11, 12, 21). Thus, suppression of apoptosis by p35 overexpression was expected to enhance the yield of BV progeny. We consistently measured a fivefold increase in BV yield of vHSP-P35, which overexpressed p35, compared to wt AcMNPV progeny yields. This reflects only a slight improvement in the ability of AcMNPV to complete a productive infection. The above result could be attributed to an unexpected second mutation in the genome of the recombinant virus vHSP-P35; however, BV yields from recombinant- and wt-infected SF9 permissive cells were similar. Another possible explanation is the low level of competence of the SL2 cells to sustain NPV replication, but we have previously reported that SINPV replicates to high titers in this cell line (9). Moreover, we report here that vHSP-P35 and wt BVs derived cell line that expresses p35 constitutively and is completely resistant to AcMNPV-induced apoptosis. Under these conditions, no dramatic improvement in BV titers of wt AcMNPV compared to those of infected neomycin-resistant control cells was observed (17a).

Thus, taken together, these data lead to the conclusion that apoptosis is not the only block to AcMNPV replication in SL2 cells, meaning that p35 is probably not the only AcMNPV host range determinant in this system (and probably at the organism level). Study of the expression of other early viral genes, such as those required for efficient late-gene expression and DNA replication (26, 38), may provide further clues to understanding the deficient replication of AcMNPV in SL2 cells.

Finally, recent studies have indicated that Choristoneura fumiferana MNPV (CMNPV) is able to suppress AcMNPV-induced apoptosis of C. fumiferana CF-203 cells and rescue the infectivity of AcMNPV for T. ni larvae (32). SINPV infection of SL2 cells is permissive and does not induce apoptosis (9). Coinfection experiments with AcMNPV and S. littoralis NPV may yield a permissive infection and help elucidate the mechanism of abortion of the AcMNPV infection in SL2 cells. Moreover, the availability of in vitro AcMNPV DNA replication assays (23, 26) may help define the minimal set of genes required to obtain efficient amplification of the viral genome in SL2 cells (9).

![Fig. 8: Growth curve of recombinant (vHSP-P35) and wt AcMNPVs.](http://jvi.asm.org/)

![Fig. 9: Mortality of virus-infected S. littoralis larvae.](http://jvi.asm.org/)
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ADDITIONAL

SF9 (subclone of SF21) cells are as susceptible to apoptosis by the AcMNPV p35 null mutant α-A35KlacZ as SF21 cells.

REFERENCES