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Site-Specific Mutagenesis of the 35-Kilodalton Protein Gene Encoded by *Autographa californica* Nuclear Polyhedrosis Virus: Cell Line-Specific Effects on Virus Replication

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The gene encoding the 35-kDa protein (35K gene) located within the *Eco*RI-S genome fragment of *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) is transcribed early in infection. To examine its function(s) with respect to virus multiplication, we introduced specific mutations of this early gene into the Ac*M*NPV genome. In *Spodoptera frugiperda* (SF21) culture, deletion of the 35K gene reduced yields of extracellular, budded virus from 200- to 15,000-fold, depending on input multiplicity. Mutant replication was characterized by dramatically diminished levels of late and very late (occlusion-specific) virus gene expression and premature cell lysis. In contrast, 35K gene inactivation had no effect on virus growth in cultured *Trichoplusia ni* (TN368) cells. Insertion of the 35K gene and its promoter at an alternate site (polyhedrin locus) restored virus replication to wild-type levels in SF21 culture. Subsequent insertion of 4 bp after codon 81 generated a frameshift mutant that exhibited a virus phenotype indistinguishable from that of 35K deletion mutants and demonstrated that the 35K gene product (p35) was required for wild-type replication in SF21 cells. Mutagenesis also indicated that the C terminus of p35, including the last 12 residues, was required for function. In complementation assays, wild-type virus bearing a functional 35K gene allele stimulated all aspects of 35K null mutant replication and suppressed early cell lysis. These findings indicated that p35 is a *trans*-dominant factor that facilitates Ac*M*NPV growth in a cell line-specific manner.

Early genes encoded by *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV), the prototype of the subgroup A baculoviruses, are of interest because they influence important regulatory processes of the virus and the host insect during infection. Early Ac*M*NPV genes include those involved in transactivation of virus gene expression (IE0, IE1, and IE-N), virus DNA replication (DNA polymerase, *pcna* [proliferating cell nuclear antigen], and DNA helicase), and suppression of insect metamorphosis (*egt* [ecdysteroid UDPglucosyltransferase]) (2, 4, 12, 17, 22, 27, 34). The expression of early genes from the 128-kb Ac*M*NPV genome requires host RNA polymerase II and initiates prior to virus DNA synthesis. In contrast, expression of late and very late genes depends on a distinct RNA polymerase and DNA replication (for reviews, see references 1, 6, and 28). Late virus genes include those involved in the production of virus progeny that are composed of two infectious forms: enveloped virions that bud from the plasma membrane (budded virus [BV]) and occluded virus particles that accumulate later in the nucleus of the host cell prior to lysis that occurs from 60 to 90 h after infection. Since proper expression of early virus genes is required for subsequent late gene expression, they play a critical role in virus replication and may function as determinants of Ac*M*NPV host range.

We have investigated the role of the early 35-kDa protein gene (35K gene) encoded by the *Eco*RI-S genome fragment of Ac*M*NPV (86.8 to 87.9 map units [m.u.]) in facilitating virus replication in a cell line-specific manner. Our interest in the function and regulation of the 35K gene originated with the finding that the nearby insertion of a host-derived retrotransposon dramatically altered transcription within the region (8, 9, 24). To initiate studies of the possible function(s) of the affected genes, including the 35K gene, we have constructed deletion (null) mutants of Ac*M*NPV and examined the effect of such mutations on virus replication processes. The 35K gene encodes a 35-kDa polypeptide (p35), as demonstrated by in vitro translation of hybrid-selected mRNA from Ac*M*NPV-infected cells (7). Characteristic of early Ac*M*NPV genes, the predominant mRNA (α_1) for p35 is detected within the first hour after infection, accumulates through 6 to 8 h, and declines thereafter (25). Host RNA polymerase II-mediated transcription is controlled by an early virus promoter (composed of a TATA sequence and RNA start site) that is influenced by regulatory motifs located immediately upstream (5, 16, 25). A weaker late-promoter element is located near the early RNA start site (25) and may function to extend 35K gene expression into the later stages of infection.

Recent identification of a spontaneous Ac*M*NPV deletion mutant and subsequent characterization of a site-specific deletion mutant by Clem et al. (3) indicated that loss of sequences within the 35K open reading frame (ORF) causes premature lysis and death of infected host cells. This virus-induced phenomenon was cell line specific, since cultured *Spodoptera frugiperda* (SF21) cells were susceptible to lysis whereas *Trichoplusia ni* (TN368) cells were not. The early lysis of SF21 cells was characterized as that resulting from programmed cell death (apoptosis) on the basis of stereotypic changes in cell morphology and degradation of nuclear DNA into oligonucleosome-sized fragments. This indicated that a function of the 35K gene is to directly or indirectly suppress virus-induced apoptosis (3), a process that may be an antiviral defense mechanism by the host (23, 32, 37). The

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exact role of the 35K gene product (p35) has yet to be determined.

In this report, we describe site-directed mutagenesis of Ac*M*NPV to demonstrate that p35 is required for wild-type levels of virus replication, but only in apoptosis-sensitive cells. 35K null mutants exhibited a dramatic growth disadvantage compared with wild-type virus. Moreover, these mutants exhibited highly restricted levels of late and very late virus gene expression. These defects were consistent with the loss of virus DNA, possibly through the activation of host nucleases during apoptosis. 35K null mutants were also *trans* complemented by a functional 35K allele supplied by coinfection with wild-type virus, demonstrating the dominant nature of this gene. p35 is thus the first Ac*M*NPV gene product identified that provides a selective growth advantage in a cell line-specific manner.

MATERIALS AND METHODS

Viruses and cells. The L-1 strain of Ac*M*NPV (20) and indicated virus mutants were propagated in established *S. frugiperda* IPL-SF21 (35) and *T. ni* TN368 (14) cell lines with TC100 growth medium (GIBCO Laboratories) supplemented with 2.6 mg of tryptose broth per ml and 10% heat-inactivated fetal bovine serum (HyClone Laboratories). At time zero, cell monolayers were inoculated with extracellular BV. After a 1-h adsorption, the residual inoculum was replaced with growth medium. The infected cells were then incubated at 27°C.

Plasmid DNAs and transplacement vectors. A transplacement plasmid was constructed for each Ac*M*NPV recombinant generated. For deletion mutant vΔ35K, the 5.37-kb *Bam*HI-*Sst*I fragment of the Ac*M*NPV genome (84.4 to 88.1 m.u.) was first cloned into vector pUC19, producing plasmid pBB/BSst. Plasmid pΔ35K, in which the 35K ORF was deleted, was generated by *Nru*I and *Spe*I digestion of pBB/BSst, end repair with the Klenow fragment of DNA polymerase I, addition of an 8-bp *Bgl*II linker, and intramolecular ligation. Transplacement plasmids for recombinant viruses in which the 35K gene (or mutations thereof) was inserted at the polyhedrin locus (3.96 m.u.) were constructed from vectors containing the *Escherichia coli lacZ* gene. In brief, a 3.3-kb fragment containing the *lacZ* gene under control of the polyhedrin promoter was inserted into the unique *Xho*I and *Kpn*I sites of pEVocc$^+$/PA (5), generating plasmid pEV-lacZ. The *lacZ* fragment was previously excised from plasmid pPOLY-lacZ in which the *lacZ* gene from pMC-1871 (33) was inserted downstream from the polyhedrin promoter cloned into the pBluescript (KS) vector (Stratagene). Next, the 35K gene under control of its own promoter was inserted into pEV-lacZ. This was accomplished by first constructing plasmid p35K-ORF through insertion of a 1.28-kb *Mlu*I-*Eco*RI fragment (end repaired at the *Mlu*I site), containing the entire 35K ORF and promoter sequences, at *Hin*cII and *Eco*RI sites, respectively, of pBluescript. The 35K gene fragment was then excised by digestion with *Xho*I and *Xba*I and inserted into the corresponding sites of pEV-lacZ to generate plasmid pEV-lacZ/35K$^+$. pEV-lacZ/35K$^{Bcl\text{-}fs}$ was constructed by *Bcl*I digestion of p35K-ORF, end repair with the Klenow fragment, blunt-end ligation, and insertion of the altered 35K gene fragment into pEV-lacZ, as described above. pEV-lacZ/35K$^{\Delta Spe}$ was generated by *Spe*I digestion of pEV-lacZ/35K$^+$, followed by end repair with the Klenow fragment and blunt-end ligation.

Recombinant viruses. Standard gene replacement methods (28, 36) were used to construct Ac*M*NPV recombinants. To generate viruses wt/lacZ and vΔ35K, 2×10^6 SF21 cells were transfected with transplacement plasmids pEV-lacZ and pΔ35K, respectively, along with wild-type Ac*M*NPV DNA by using Lipofectin (Bethesda Research Laboratories) as described previously (5). Recombinants vΔ35K/lacZ, vΔ35K/lacZ/35K$^+$, and related viruses lacking the 35K gene in its native position (87 to 88 m.u.) were generated in the same way, except that viral DNA was replaced by an equivalent volume of extracellular vΔ35K virus containing approximately 5×10^5 PFU. The later viruses were identified and plaque purified with TN368 monolayers on the basis of their occlusion-negative phenotype and production of β-galactosidase that was visualized by including 150 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml in the agarose overlay. Deletion mutant vΔ35K was identified on the basis of its occlusion-negative plaque phenotype by using SF21 cells. Proper insertion or deletion of sequences was ascertained by restriction mapping and Southern blot analysis of isolated DNA from each recombinant virus (data not shown). All virus titers were obtained by standard plaque assays with TN368 cells. In the case of *lacZ*-containing viruses, blue plaques were counted 4 days after infection in the presence of X-Gal.

Analysis of virus protein synthesis. At the indicated times after infection, the medium above SF21 or TN368 cell monolayers (10^6 cells per plate) was removed and replaced with phosphate-buffered saline (pH 6.2) (20) containing 200 μCi of Trans^{35}S-Label (1,200 Ci/mmol, methionine ≥70%, cysteine ≤15%; ICN Biomedicals, Inc.) per ml. After a 1-h incubation at 27°C, the cells were dislodged, collected by centrifugation (500 × *g* for 5 min), and lysed with 1% sodium dodecyl sulfate (SDS) and 2.5% β-mercaptoethanol. Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (19) and autoradiography.

β-Galactosidase assays. Infected SF21 or TN368 cells were dislodged, collected by centrifugation (500 × *g* for 5 min), and washed with ice-cold phosphate-buffered saline (pH 6.2). The cell pellets were suspended in 0.25 M Tris (pH 8.0) and subjected to three freeze-thaw cycles. Clarified extracts (16,000 × *g*) were assayed immediately for β-galactosidase with the substrate *p*-nitrophenyl-β-D-galactopyranoside essentially as described elsewhere (31). When necessary, extracts were diluted with 0.25 M Tris (pH 8.0) to ensure linearity of the assay.

Dot blot hybridizations. SF21 monolayers (10^6 cells) were harvested 48 h after infection as described above. To isolate total intracellular DNA, the cells were suspended in 10 mM Tris (pH 8.0)–1 mM EDTA and incubated in 0.2% SDS and 0.1 mg of proteinase K per ml for 2 to 4 h at 37°C. Protein and RNA were removed by phenol extraction and treatment with 120 μg of RNase A per ml, respectively. DNA (from 10^5 cells) was heat denatured (10 min, 100°C), snap cooled on ice, and mixed with an equal volume of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). With a dot blot apparatus, the entire sample was applied to a Hybond-N (Amersham) nylon membrane and prepared for hybridization as prescribed by the manufacturer. Blotted DNA was hybridized to specific DNA probes at 65°C for 16 h. An [α-^{32}P]dATP-labelled, random-primed probe (Amersham) consisting of a pBluescript plasmid containing only *lacZ*-specific sequences was used to monitor intracellular levels of vΔ35K/lacZ virus DNA. After the blots were washed, the extent of probe hybridization was determined by measuring radioactivity associated with individual spots with a Betascope 603 Blot Analyzer. The linearity of the assay was demonstrated by simultaneously including increasing

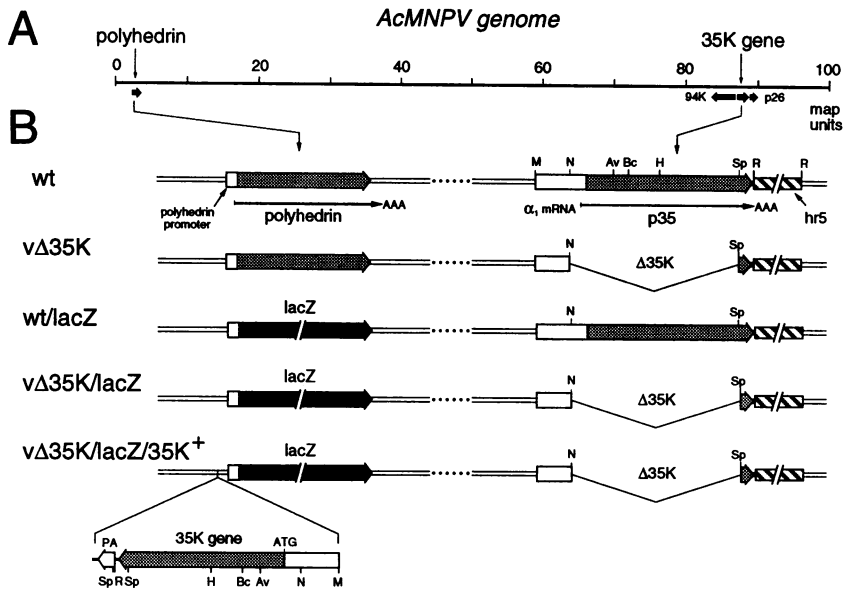


FIG. 1. Genome organization of wild-type and 35K gene mutants of Ac*M*NPV. (A) Polyhedrin and 35K genes on the Ac*M*NPV linear map. Vertical arrows depict the positions of the polyhedrin (4.3 m.u.) and 35K protein (87.4 m.u.) genes; the 35K gene is flanked on either side by the 94K protein and p26 genes. (B) Polyhedrin and p35 locus of wild-type (wt) virus and 35K gene mutants. Virus designations indicate the presence or absence of 35K and *lacZ* genes within the viral genome; protein coding regions are depicted by the shaded arrows. vΔ35K deletion mutants lack the *Nru*I-*Spe*I fragment that includes the promoter and all but 12 codons of the 35K ORF. Viruses wt/lacZ, vΔ35K/lacZ, and vΔ35K/lacZ/35K$^+$ contain the *lacZ* gene (under control of the polyhedrin promoter) in place of the structural portion of the polyhedrin gene. Virus vΔ35K/lacZ/35K$^+$ contains the 35K gene and its promoter (on a 1.28-kb *Mlu*I-*Eco*RI fragment derived from the *Eco*RI-S region) inserted adjacent (3.96 m.u.) to the *lacZ* gene and upstream from a polyadenylation signal (PA). The α_1 mRNA (1.1 kb) for p35 initiates 26 bp upstream from the predicted initiator codon (ATG) and is polyadenylated 90 bp upstream from the leftmost *Eco*RI site of the hr5 enhancer (striped box). Relevant restriction sites of the 35K gene fragment are indicated. Av, *Ava*II; Bc, *Bcl*I; H, *Hin*dIII; M, *Mlu*I; N, *Nru*I; R, *Eco*RI; Sp, *Spe*I.

amounts of membrane-bound *lacZ* plasmid DNA in the hybridization protocol.

RESULTS

Construction of 35K gene null mutations. In preliminary experiments designed to investigate the function(s) of the 35K protein gene, we examined the effect of null mutations on Ac*M*NPV replication by constructing recombinant viruses in which the sequences that encompass the 35K gene were deliberately deleted. To facilitate identification of such mutants, we inserted the *E. coli lacZ* gene into the 35K ORF (between the *Ava*II and *Spe*I sites). This generated a potential fusion protein in which the N-terminal 51 amino acids of p35 were linked to β-galactosidase and simultaneously removed 702 bp of the 35K ORF. When propagated in *S. frugiperda* (SF21) monolayers, the resulting 35K-lacZ virus recombinants exhibited a plaque morphology that was distinct from that of wild-type virus on the basis of smaller size and lack of occlusion bodies; low-level *lacZ* expression was indicated by light-blue plaques in the presence of X-Gal. A similar plaque phenotype was exhibited by an independently constructed Ac*M*NPV mutant, vΔ35K (Fig. 1), in which 950 bp of DNA sequences between the *Nru*I and *Spe*I sites were deleted, thereby removing the 35K promoter and 287 (of 299) codons from the 35K ORF. Both deletion mutants produced unusually low yields of BV in SF21 cultures. In contrast, when propagated in *T. ni* (TN368) cells, mutant virus yields were similar to that of wild-type virus (see below). Moreover, vΔ35K and wild-type virus plaques were indistinguishable, as judged by relative size and yield of occluded virus.

To more accurately examine the growth properties of the 35K null mutants in both lepidopteran cell lines, we constructed a series of 35K ORF mutants in which the structural portion of the polyhedrin gene was replaced by the *lacZ* gene (Fig. 1). Virus wt/lacZ contains the wild-type 35K ORF at its native position (*Eco*RI-S genome fragment), while sequences between the *Nru*I and *Spe*I sites of the 35K ORF were deleted in virus vΔ35/lacZ. Virus vΔ35K/lacZ/35K$^+$ contains a single copy of the wild-type 35K ORF and its promoter inserted adjacent to the *lacZ* gene (at the polyhedrin locus). Quantitation of β-galactosidase activity in cells infected by these viruses provided a means to monitor late (occlusion-specific) gene expression. In addition, visualization of blue plaques on TN368 monolayers provided an accurate and reliable measure of infectious BV from 35K null mutants. Since mutant and wild-type viruses exhibited identical growth properties in TN368 cells (see below), all titers in this report are defined as PFU produced on TN368 monolayers.

Sequences that include the 35K ORF are required to maintain wild-type production of BV. To directly compare the growth properties of 35K null mutants, we measured the yields of progeny BV from SF21 and TN368 cultures inoculated with increasing amounts of virus. Since multiple rounds of infection were possible at the lowest multiplicities of infection (MOIs), the yield of BV represented that accumulated over the 48-h period examined and provided an indirect measure of the replication competence of the viruses. In SF21 cells (Fig. 2A), virus vΔ35K/lacZ produced 200- to 15,000-fold less BV than wt/lacZ. The greatest difference (15,000-fold) was observed with an input of 0.1 PFU per cell (Table 1). In contrast to wt/lacZ, the yield of vΔ35K/lacZ increased steadily with the MOI used; a 500-fold increase in MOI (from 0.01 to 5 PFU/cell) yielded a 370-fold

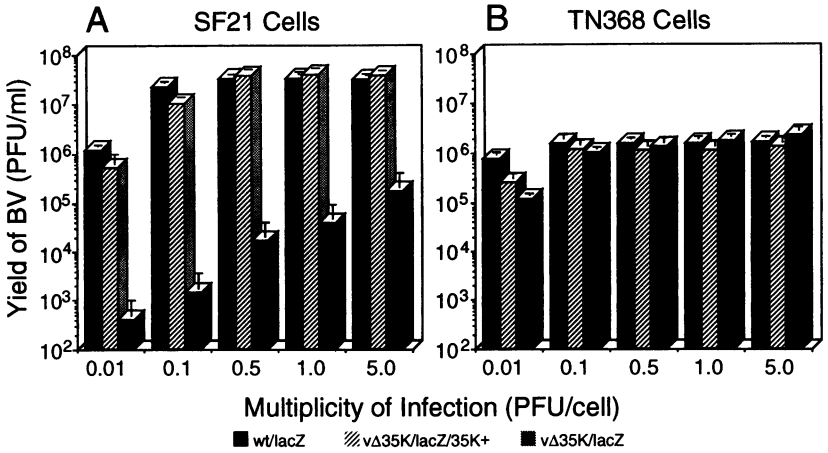


FIG. 2. Production of BV by 35K gene mutants. SF21 (A) and TN368 (B) monolayers (1.8×10^6 cells per plate) were inoculated with viruses wt/lacZ, vΔ35K/lacZ/35K$^+$, and vΔ35K/lacZ at the indicated MOIs (PFU per cell). After a 1-h adsorption, the cells were washed, covered with growth medium (5 ml), and incubated at 27°C. The extracellular medium was harvested 48 h after infection, and the yield of BV was determined by plaque assay with TN368 cells. The plaques were visualized by including X-Gal in the agarose overlay. BV yields are reported as PFU per milliliter with a logarithmic scale; error bars for each value are shown.

increase in BV. Consequently, the ratio of wt/lacZ BV to vΔ35K/lacZ BV decreased with increases in the MOI (Table 1). One-step growth studies indicated that while progeny BV from 35K null mutants appeared at the same time as wild-type BV (24 h after infection), overall levels were reduced throughout infection (0 to 96 h) (21). Visual inspection of vΔ35K/lacZ-infected SF21 cells also indicated that the extent of early cell lysis (characteristic of 35K null mutants) was proportional to the amount of input virus. Lysis resembled that described by Clem et al. (3), including plasma membrane blebbing, progressive cell disintegration, and concomitant degradation of cell DNA into oligonucleosome-sized fragments (data not shown). Lysis of wt/lacZ-infected cells was not observed until 72 to 96 h after infection and did not include cell blebbing.

In contrast, the yield of BV from TN368 cells infected with the 35K null mutant vΔ35K/lacZ was comparable to that of wild-type virus, wt/lacZ (Fig. 2B). Only at the lowest MOI was a significant difference detected; with 0.01 PFU/cell, vΔ35K/lacZ produced approximately sevenfold less BV than wt/lacZ (Table 1). No early cell lysis was detected in TN368 cultures upon inoculation with vΔ35K/lacZ or other 35K null mutants.

BV production was restored to wild-type levels (Fig. 2) when the 35K gene was inserted back into the genome of vΔ35K/lacZ, but at an alternate location (polyhedrin locus [3.96 m.u.]). vΔ35K/lacZ/35K$^+$ yields were comparable to that of wt/lacZ in both cell lines. Only at the lowest MOI (0.01 PFU/cell) did vΔ35K/lacZ/35K$^+$ exhibit a growth disadvantage in SF21 or TN368 cells (respectively, 2.3- and 2.9-fold lower than that of wt/lacZ) (Table 1). The cytopathic effect of vΔ35K/lacZ/35K$^+$ in SF21 cells was also indistinguishable from that of wild-type virus wt/lacZ. Thus, the 1.28-kb *Mlu*I-*Eco*RI fragment that encompasses the 35K ORF and its promoter (Fig. 1) contains the information necessary to restore the wild-type virus phenotype in SF21 culture.

35K null mutants exhibit restricted levels of late virus gene expression. The absence of occluded virus particles in vΔ35K-infected SF21 cells suggested that deletion of the 35K ORF resulted in loss of late virus gene expression. To quantitate this effect, we used the *lacZ* gene under control of the polyhedrin promoter to monitor levels of very late (occlusion-specific) gene expression. In SF21 cells, mutant vΔ35K/lacZ produced only low levels of β-galactosidase at all times examined (Fig. 3). In contrast, β-galactosidase expression in wt/lacZ-infected cells paralleled that of polyhedrin during a wild-type infection (29), reaching a maximum by 48 h and declining thereafter. At peak expression, β-galactosidase levels upon infection with wt/lacZ were 300-fold higher than that of vΔ35K/lacZ. Approximately wild-type levels of β-galactosidase were synthesized in SF21 cells by virus vΔ35K/lacZ/35K$^+$ (Fig. 3), indicating that insertion of the 35K gene and promoter back into the virus genome restored occlusion-specific expression. Identical analyses with TN368 cells indicated that β-galactosidase expression was comparable for all three viruses (Fig. 3). In general, β-galactosidase appeared earlier and reached higher levels in TN368 cells than in SF21 cells.

To examine the effect of 35K null mutations on other

TABLE 1. Comparison of BV yields for wt/lacZ, vΔ35K/lacZ, and vΔ35K/lacZ/35K$^+$

Cell line	Ratio of wt/lacZ to vΔ35K/lacZ[a] at the following MOIs (PFU/cell):					Ratio of wt/lacZ to vΔ35K/lacZ/35K$^+$[b] at the following MOIs (PFU/cell):				
	0.01	0.1	0.5	1.0	5.0	0.01	0.1	0.5	1.0	5.0
SF21	2,900	15,000	2,000	900	200	2.3	2.1	0.9	0.9	0.9
TN368	6.6	1.6	1.2	0.9	0.7	2.9	1.3	1.4	1.4	1.2

[a] The wt:vΔ35K ratio was calculated by dividing the yield (PFU) of wt/lacZ virus by the yield of vΔ35K/lacZ virus obtained at the same MOI.
[b] The wt:vΔ35K/35K$^+$ ratio was calculated by dividing the yield (PFU) of wt/lacZ virus by the yield of vΔ35K/lacZ/35K$^+$ virus obtained at the same MOI.

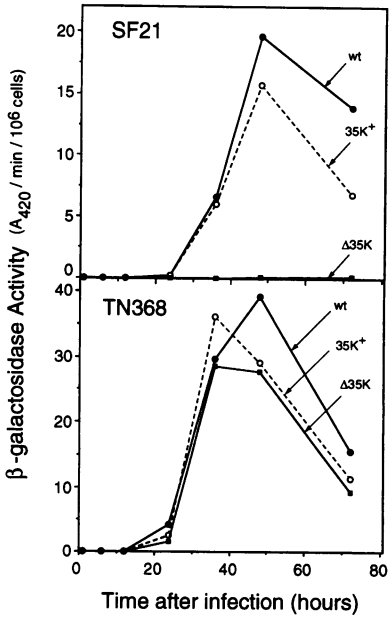


FIG. 3. Comparison of very late, occlusion-specific gene expression by wild-type and 35K null viruses. SF21 and TN368 monolayers (10^6 cells per plate) were inoculated with 5 PFU of recombinant viruses wt/lacZ (wt), vΔ35K/lacZ (Δ35K), and vΔ35K/lacZ/35K$^+$ (35K$^+$) per cell. At the indicated intervals after infection, the cells were harvested, lysed, and assayed for intracellular β-galactosidase. Measured enzyme activity is reported as A_{420} units produced per minute per 10^6 cells. The values shown are the averages of two independent experiments.

classes of AcMNPV proteins, we compared protein synthesis throughout infection in both cell lines. SF21 cells infected with viruses wt/lacZ and vΔ35K/lacZ/35K$^+$ exhibited similar protein patterns that were typical of a wild-type virus infection (Fig. 4A); this included the appearance and disappearance of early proteins (6 through 12 h), followed by late proteins (12 through 24 h), and finally very late, occlusion-specific proteins (e.g., β-galactosidase). Although early virus-induced proteins were detected in SF21 cells infected at an identical multiplicity as vΔ35K/lacZ, synthesis of late virus proteins was severely restricted (Fig. 4A). Proteins that normally appeared by 12 h in wild-type infections (during the period of viral DNA replication) were not detected or appeared later and at very low levels. In addition, host protein synthesis continued as late as 50 h after infection; the observed reduction from 36 to 50 h (Fig. 4A) was attributed to vΔ35K/lacZ-induced early cell lysis. Similar analyses (data not shown) indicated that inoculations with higher MOIs of 35K null mutants (including virus vΔ35K [Fig. 1]) caused a more rapid reduction in intracellular protein synthesis; in such cases, little synthesis was detected 36 h after infection. This reduction paralleled a more rapid induction of early cell lysis. Of the cells remaining late in infection, few viral proteins were detected amid the background of host proteins.

The pattern of virus-induced protein synthesis in TN368 cells was nearly identical for each of the three viruses wt/lacZ, vΔ35K/lacZ, and vΔ35K/lacZ/35K$^+$ (Fig. 4B). This included the characteristic reduction in host protein synthesis that began 18 h after infection for each virus. Minor differences were detected in the level of several less prominent early (6 and 12 h) proteins. Direct comparison of the

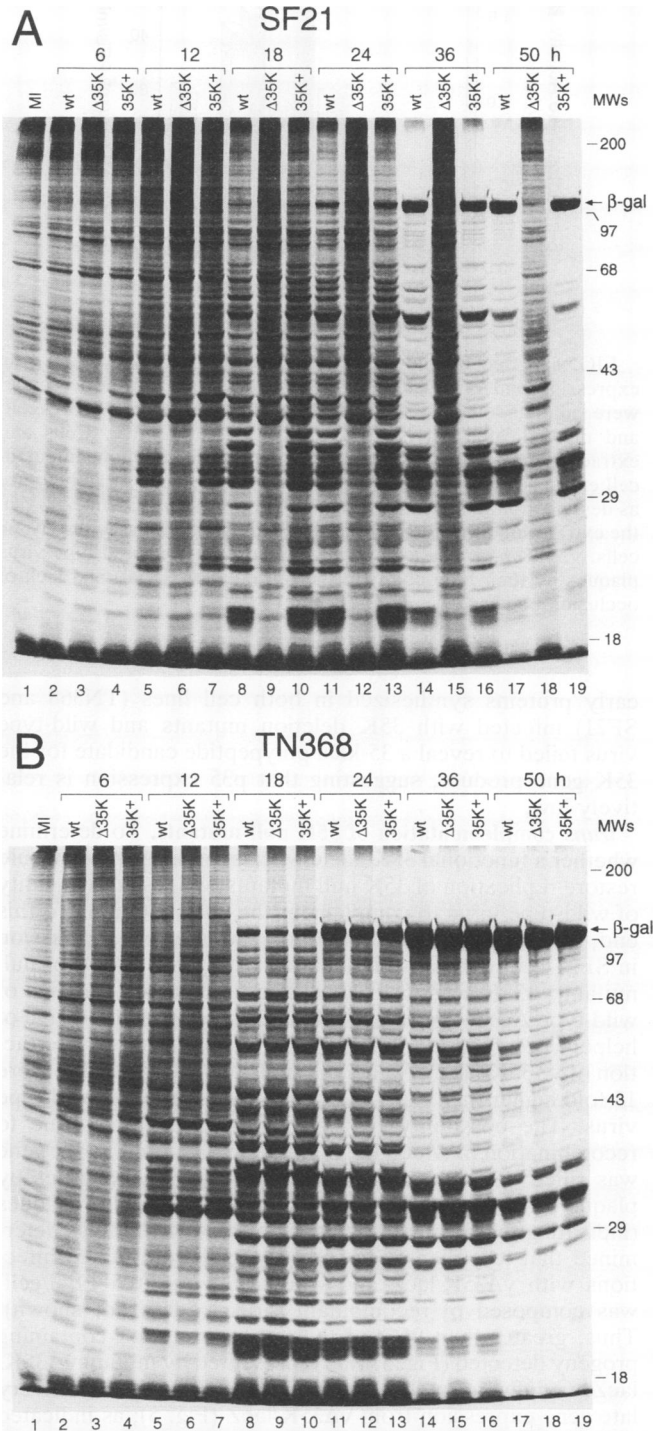


FIG. 4. Comparison of proteins synthesized in cells infected with wild-type and vΔ35K viruses. SF21 (A) and TN368 (B) monolayers were inoculated with 5 PFU of recombinant viruses wt/lacZ (wt), vΔ35K/lacZ (Δ35K), and vΔ35K/lacZ/35K$^+$ (35K$^+$) per cell. At the indicated intervals after infection, the cells were radiolabeled for 1 h with [^{35}S]methionine-cysteine, lysed, and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. Lysates prepared from cells radiolabeled after mock-infection (MI) are included (lane 1). The positions of β-galactosidase (β-gal) and molecular weight standards (MWs) are indicated (sizes in kilodaltons).

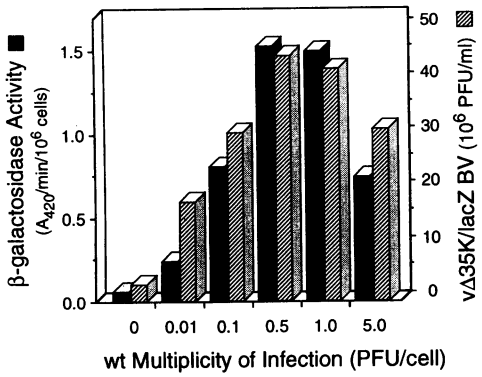


FIG. 5. Effect of wild-type virus on mutant vΔ35K/lacZ late gene expression and BV yields. SF21 monolayers (10^6 cells per plate) were simultaneously inoculated with virus vΔ35K/lacZ (3 PFU/cell) and the indicated MOIs of wild-type (wt) Ac*M*NPV. Cells and extracellular medium were harvested 48 h after infection. Infected-cell extracts were assayed for β-galactosidase; units of activity are as described in the legend to Fig. 3. The yield of vΔ35K/lacZ BV in the extracellular medium was determined by plaque assay on TN368 cells. vΔ35K/lacZ plaques were distinguished from wild-type virus plaques by their blue color (in the presence of X-Gal) and lack of occlusion bodies.

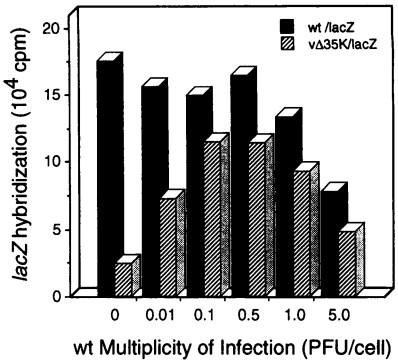


FIG. 6. Effect of wild-type virus on intracellular levels of vΔ35K/lacZ DNA. SF21 monolayers were inoculated with 3 PFU of virus vΔ35K/lacZ or wt/lacZ per cell and the indicated MOI of wild-type (wt) Ac*M*NPV. Intracellular DNA was isolated 48 h after infection. The level of *lacZ*-specific DNA was ascertained by dot blot hybridizations with a radiolabeled DNA probe containing *lacZ* sequences. The extent of hybridization was determined by measuring the radioactivity associated with individual spots and is reported as counts per minute bound.

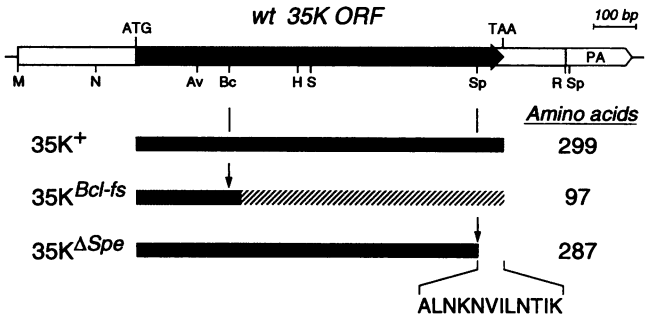
early proteins synthesized in both cell lines (TN368 and SF21) infected with 35K deletion mutants and wild-type virus failed to reveal a 35-kDa polypeptide candidate for the 35K gene product, suggesting that p35 expression is relatively low.

***trans* complementation of 35K null mutants.** To determine whether a functional 35K allele when supplied in *trans* would restore replication of 35K null mutants, we tested the ability of wild-type virus to complement the null mutation. To this end, we monitored BV production and late gene expression in SF21 cells that were inoculated with a fixed MOI of null mutant vΔ35K/lacZ (3 PFU/cell) and increasing amounts of wild-type Ac*M*NPV (Fig. 5). In a way that was indicative of helper virus function, wild-type virus enhanced the production of vΔ35K/lacZ BV; maximum levels of mutant BV were 15-fold higher than that obtained in the absence of wild-type virus. The possibility that this enhancement was due to recombination in which the null mutant acquired a 35K gene was ruled out by screening BV from mixed infections by plaque assay with SF21 cells. By scoring for blue plaques (indicating the rescue of late gene expression), it was determined that less than 5% of the progeny from mixed infections with vΔ35K/lacZ and wild-type virus (0.5 PFU/cell) was composed of recombinant viruses (data not shown). Thus, greater than 95% of the increase in *lacZ*-containing progeny detected (Fig. 5) was represented by mutant vΔ35K/lacZ. Coinfection with wild-type virus also stimulated very late gene expression from vΔ35K/lacZ (Fig. 5); as indicated by β-galactosidase levels, very late gene expression increased as much as 30-fold. At wild-type virus MOIs of greater than 1 PFU/cell, β-galactosidase expression declined. Since a similar reduction was observed in control experiments in which vΔ35K/lacZ was replaced by wt/lacZ (data not shown), we attributed this decline to competition between viruses for limiting host cell factors (e.g., virus receptors or biosynthetic machinery). Lastly, visual inspection of coinoculated cultures also indicated that the extent of early cell lysis induced by vΔ35K/lacZ decreased in proportion to the increase in wild-type virus.

Further support for *trans* complementation by wild-type virus was obtained by measuring intracellular levels of vΔ35K/lacZ-specific DNA in SF21 cells (Fig. 6). As demonstrated by hybridization to a DNA probe complementary to *lacZ* sequences, vΔ35K/lacZ DNA increased by as much as fivefold upon coinfection with wild-type virus (0.1 PFU/cell). An identical increase was observed with a different 35K null mutant (vΔ35K/lacZ/35K$^{Bcl\text{-}fs}$; see below). In contrast, wt/lacZ DNA levels declined as the wild-type virus MOI increased in similar assays (Fig. 6), suggesting again that competition for limiting factors was responsible. Thus, the level of intracellular DNA of 35K null mutants was also affected by wild-type virus containing a functional 35K allele. It remains to be determined whether this increase was due to enhanced viral DNA synthesis by the 35K null mutants or to a reduction in virus DNA loss that resulted from suppression of early cell lysis.

p35 nonsense mutants exhibit the 35K null phenotype. To verify that the 35K null phenotype was due to inactivation (or loss) of the 35K gene product (p35) and not to the removal of *cis*-acting regulatory or replication signals resulting from the deletion of sequences from the mutants described above, we tested the effect of additional 35K ORF mutations on virus multiplication. Since restricted late gene expression was characteristic of the 35K null phenotype, β-galactosidase expression from the polyhedrin promoter was used to monitor virus replication.

First, a nonsense mutation was introduced within the N-terminal third of the 35K ORF through the insertion of 4 nucleotides at the *Bcl*I site (Fig. 7). This resulted in a frameshift that truncated p35 after 97 amino acid residues without altering sequences downstream. Intracellular expression of β-galactosidase by the resulting virus vΔ35K/lacZ/35K$^{Bcl\text{-}fs}$ was comparable to that of the deletion mutant vΔ35K/lacZ (Fig. 7); expression in SF21 cells was approximately 100-fold lower than that of 35K gene-containing viruses (wt/lacZ and vΔ35K/lacZ/35K^{+}) but was nearly equivalent to that of the two viruses in TN368 cells. The *Bcl*I-frameshift mutant also induced early cell lysis (12 to 24 h) in SF21 cultures that was indistinguishable from that of 35K deletion mutants (data not shown). Thus, generation of a frameshift mutation with minimal sequence alterations



Virus	β-galactosidase Activity (A_{420}/min/10^6 cells) TN368	SF21	SF/TN Activity (Percent of 35K+)
wt/lacZ	46.9 ± 2.0	4.7 ± 0.6	108
vΔ35K/lacZ/35K+	41.9 ± 6.3	3.9 ± 0.3	100
vΔ35K/lacZ/35KBcl-fs	38.0 ± 1.6	0.040	1.1
vΔ35K/lacZ/35KΔSpe	51.9 ± 2.7	0.065	1.3
vΔ35K/lacZ	29.7 ± 3.4	0.060	2.2

FIG. 7. Site-directed mutagenesis of p35. The wild-type (wt) 35K ORF (shaded arrow) encodes 299 amino acid residues (from ATG to TAA codons). Virus vΔ35K/lacZ/35K+ (35K+) contains the full-length ORF (shaded box) inserted immediately upstream from a polyadenylation signal (PA) at the polyhedrin locus (Fig. 1). The inserted 35K ORF of virus vΔ35K/lacZ/35KBcl-fs (35KBcl-fs) contains a 4-bp insertion at the unique *BclI* site, causing a frameshift that truncates p35 after 81 residues and fuses 16 out-of-frame residues (black box) to the C terminus; the hatched box depicts the remaining sequences of the ORF. The truncated 35K ORF (287 residues) of virus vΔ35K/lacZ/35KΔSpe (35KΔSpe) was generated by the introduction of a nonsense mutation at the *SpeI* site and removal of the 12 C-terminal amino acid residues as shown (7). Small vertical arrows mark the end of the 35K ORF for both mutants. Restriction site abbreviations are listed in Fig. 1. Intracellular β-galactosidase activity was determined 48 h after infection of SF21 or TN368 cultures, with an MOI of 1 PFU/cell. Values for β-galactosidase activity are the averages of duplicate infections. The activity ratio of SF21 to TN368 was calculated by normalizing the ratio of activity in SF21 and TN368 cells of the indicated viruses to that of vΔ35K/lacZ/35K+ (35K+), defined as 100%.

(4-bp insertion) was sufficient to cause the null phenotype and demonstrated that p35 is required for wild-type virus replication.

This conclusion was supported by the construction of a p35 truncation mutant in which the last 12 C-terminal residues were removed from the 35K ORF (Fig. 7). A nonsense codon was introduced at residue 288 by deleting the 145-bp region between the *SpeI* and *EcoRI* sites of the 35K gene fragment also inserted at the polyhedrin locus. Compared with other 35K mutants, virus vΔ35K/lacZ/35KΔSpe also directed synthesis of low levels of β-galactosidase in SF21 cells, but it directed wild-type levels in TN368 cells. vΔ35K/lacZ/35KΔSpe also induced premature lysis of SF21 cells. These results suggested that the C terminus, including the last 12 amino acid residues, is essential to p35 function or protein stability.

DISCUSSION

AcMNPV p35 null mutants exhibit cell line-specific growth restrictions. Our initial approach to identifying the function of genes encoded by the *Hin*dIII-K–*Eco*RI-S region of AcMNPV was to generate null mutants in which a specific gene was deleted (or replaced). Removal of various portions of the 35K ORF produced AcMNPV mutants that exhibited altered growth properties that were cell line specific. In cultured *S. frugiperda* (SF21) cells, production of BV by deletion mutant vΔ35K/lacZ was significantly reduced, ranging from 200 to 15,000 times less than that of wild-type virus, depending on the input MOI (Table 1). In contrast, yields of BV from 35K null mutants were indistinguishable from that of wild-type virus in cultured *T. ni* (TN368) cells. 35K null mutants also exhibited severely restricted levels of late gene expression in SF21 but not TN368 cultures. For example, expression of the *lacZ* gene under control of the very late polyhedrin promoter was 300-fold lower in SF21 cells infected with 35K null mutants than with wild-type virus (Fig. 3). Similar reductions in the synthesis of other late and very late proteins were detected in 35K null mutant-infected SF21 but not TN368 cells (Fig. 4).

By constructing a series of AcMNPV recombinants, including a frameshift mutant within the 35K ORF, we demonstrated that the growth restriction of the null mutants was due to loss of function of the 35K gene product (p35). Combined with the results of Clem et al. (3), these data provide strong evidence that the null phenotype is not due to loss of essential *cis*-acting DNA sequences (including regulatory or replication signals). This conclusion was supported by the finding that a functional 35K allele *trans* complemented 35K null mutants, as demonstrated by mixed infections in which wild-type virus reversed the mutant growth restrictions and stimulated BV production and very late gene expression (Fig. 5). Insertion of a 1.28-kb fragment containing the 35K ORF and its promoter at an alternative site (polyhedrin locus) restored late gene expression and BV production to wild-type levels in SF21 cells (Fig. 2 to 4). Thus, these sequences contained sufficient information (including *cis*-acting regulatory signals) to suppress the null phenotype. The finding that expression was independent of position within the virus genome, in addition to the dramatic growth advantage conferred by p35, has contributed to the development of the 35K gene as a useful selectable marker for in vitro isolation of recombinant viruses (21).

Role of p35 in AcMNPV replication. Clem et al. (3) recently demonstrated that 35K deletion mutants cause premature lysis of cultured SF21 cells that is the result of apoptosis (programmed cell death). This indicated that p35 is required to block the apoptotic process induced in response to infection by AcMNPV. Virus-induced apoptosis was distinguished by stereotypic degradation of intracellular DNA into oligonucleosome-sized fragments and premature cell lysis that involves progressive membrane blebbing. The reduced levels of 35K null mutant replication reported here may therefore be the combined effect of early cell lysis and other processes associated with apoptosis. Indeed, *trans*-complementation assays that used wild-type virus demonstrated a direct correlation between the suppression of early cell lysis and the levels of 35K null mutant replication (Fig. 5). In a way that is analogous to cytotoxic T-cell killing of virus-infected vertebrate cells in which apoptosis causes self-destruction of the target cell and prelytic fragmentation of virus DNA (23, 32), apoptosis in SF21 cells may also limit AcMNPV replication through direct fragmentation or inactivation of virus DNA. The nearly normal pattern of early protein synthesis followed by the severely restricted levels of late and very late proteins in 35K mutant-infected cells (Fig. 3 and 4) is consistent with loss of viral DNA, since late

gene expression depends on virus DNA replication (10, 30). The wild-type pattern of gene expression by these mutants in TN368 cells that are resistant to virus-induced apoptosis is also consistent with this possibility (Fig. 4). Nonetheless, it has not been ruled out that p35 directly regulates late gene expression or virus DNA replication in a cell line-specific manner.

Our results suggest that another function of p35 is to enhance the infectivity of BV. Compared with wild-type virus, significantly higher MOIs of 35K null mutants were required to induce cytopathic effects in SF21 cells. For example, when inoculated with a moderate MOI of vΔ35K/lacZ (5 PFU/cell), a large fraction of SF21 cells exhibited continued growth as indicated by increased host protein synthesis and lack of early lysis (Fig. 4). In contrast, an identical MOI of wild-type virus induced cytopathic effects and host protein shutoff in all cells. Consistent with a reduction in infectivity, increasing multiplicities of input virus produced a proportional increase in vΔ35K/lacZ progeny, whereas yields of wild-type virus remained relatively constant even at high multiplicities (Fig. 2A). 35K null mutants propagated in TN368 culture and subsequently assayed in SF21 cells exhibited similar properties, indicating that the defect was a direct consequence of the absence of p35 and not an indirect effect of the apoptotic response (i.e., fragmentation of packaged virus DNA) (18). Whether virus attachment, penetration, or uncoating is affected remains undetermined. The observation that 35K mutant replication was restored by complementation with wild-type virus, however, indicated that the lower infectivity was not solely responsible for reduced virus growth.

DNA viruses that counteract host antiviral defenses and prolong cell survival. The suppression of cell lysis and the resulting growth advantage conferred by p35 in tissue culture suggest that this protein functions to block virus-induced apoptosis within the host organism, thereby counteracting an insect antiviral defense (3). It has recently been shown that adenovirus encodes several proteins that function to evade host immunosurveillance by blocking the effects of apoptosis: gp19K blocks the transport of class I antigens of the major histocompatibility complex to the surface of adenovirus-infected cells, thereby preventing lysis by cytotoxic T-lymphocytes, while the 14.7K E3 and 19K E1B proteins protect virus-infected cells from apoptotic lysis by tumor necrosis factor (37, 38). Latent membrane protein 1 of Epstein-Barr virus also promotes survival of infected B lymphocytes by increasing expression of the host oncogene *bcl-2* (11, 13), which in turn suppresses apoptosis in these cells (15, 26). While the molecular mechanisms by which these proteins function remain undetermined, their importance to virus survival is suggested by the finding that both vertebrate and invertebrate pathogens have evolved strategies to counteract apoptosis.

An important question is whether p35 interacts directly with host components or whether it regulates other virus-encoded gene products that suppress the host response. Our studies indicate that the C-terminal 12 amino acid residues of p35 are critical for protein function or stability (Fig. 7). The C-terminal domain has a net positive charge due to a high proportion of lysine residues (7) and may therefore be important for interaction with other proteins or for targeting p35 to a specific location in the cell. Defining the interaction of p35 with other proteins as well as identifying essential protein domains should provide insight into the mechanisms by which this protein enhances Ac*M*NPV growth and infectivity.

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