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# Influence of Infection Route on the Infectivity of Baculovirus Mutants Lacking the Apoptosis-Inhibiting Gene *p35* and the Adjacent Gene *p94*

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**The infectivity of *Autographa californica* nuclear polyhedrosis virus mutants lacking the apoptosis-inhibiting gene *p35* is decreased 1,000-fold or more in larvae of the insect *Spodoptera frugiperda* if the budded form of the virus is administered by hemocoelic injection; this decrease is correlated with the antiviral effects of apoptosis (R. J. Clem and L. K. Miller, J. Virol. 67:3730–3738, 1993). We have extended this correlation by showing that the infectivity of *p35* mutant budded virus is restored to wild-type levels by expression of an unrelated baculovirus apoptosis-inhibiting gene, Cp-*iap*. We have also examined the oral infectivity of the occluded form of mutants lacking *p35*, the neighboring *p94* gene, or both genes by feeding insects occluded virus. The oral infectivity of the *p35* mutant was significantly reduced in *S. frugiperda* larvae, but this reduction (25-fold) was less than that observed for the hemocoelic route of infection (1,000-fold). The disruption of *p94* alone had no apparent effect on infectivity by either route. Unexpectedly, however, the disruption of both *p35* and *p94* restored oral infectivity to nearly wild-type levels but did not exert this compensatory effect on infectivity by hemocoelic injection. Thus, the infectivity of the double *p35*/*p94* mutant is affected in a route-specific manner in *S. frugiperda* larvae, suggesting a tissue-specific response to *p35* and/or *p94*. Infectivity in a different host, *Trichoplusia ni*, was unaffected by all the mutants tested, consistent with previous studies indicating a lack of sensitivity to apoptosis in this species. However, *T. ni* and *S. frugiperda* larvae infected with *p35* mutants failed to exhibit the symptom of morphological disintegration ("melting") typical of a wild-type infection, suggesting that *p35* is required for the infection of some tissues in both species.**

Apoptosis is triggered in the cell line SF-21, derived from the fall armyworm *Spodoptera frugiperda*, when cells are infected with *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) mutants lacking the *p35* gene (2). The apoptotic response of cells to infection has drastic effects on virus replication and gene expression, including a significant reduction in budded virus (BV) production and a block in occluded virus (OV) production (2, 11). The infectivity of *p35* mutant viruses is also greatly reduced in *S. frugiperda* larvae; if BV is administered by hemocoelic injection, the dose of BV required for 50% lethality ($LD_{50}$) is approximately 1,000-fold higher for *p35* mutants than for viruses containing *p35* (3).

The reduced in vivo infectivity of viruses lacking *p35* correlates with the inability of these mutants to block apoptosis in cell culture. The expression of *p35* in the absence of other baculovirus gene products is able to protect SF-21 cells (4) as well as mammalian neuronal cells (17) from death by apoptosis which has been induced by nonviral stimuli (e.g., actinomycin D in SF-21 cells and growth factor withdrawal or calcium ionophores in mammalian neuronal cells). In addition, while *p35* mutants induce apoptosis in SF-21 cells, they do not induce apoptosis in the permissive cell line TN-368 (2, 3, 11), derived from the cabbage looper *Trichoplusia ni*, and the infectivity of *p35* mutants is normal in *T. ni* larvae (3). Finally, in those larvae which die from *p35* mutant infection, the yield of OV is reduced 900-fold for *S. frugiperda* larvae but only 4-fold for *T. ni* larvae (3); OV production in cells undergoing apoptosis is blocked (2, 3, 6), and thus it is likely that the decreased yields of OV reflect the extent of apoptosis occurring during infection.

The region of the Ac*M*NPV genome which contains the *p35* gene (Fig. 1) (8) is of additional interest for several reasons. Located immediately upstream of *p35* in the Ac*M*NPV genome is the gene *p94* (8), which is the site of insertion for a host-derived retrotransposon in a mutant, FP-D (9, 14). In addition, both *p35* and *p94* are absent from the genome of the related baculovirus *Orgyia pseudotsugata* NPV, despite the relatively colinear organization of these two genomes (10). Thus, we were interested in testing the effect of disrupting one or both of these neighboring genes on virus replication and infectivity. While larvae can be readily infected by injection of BV into the hemocoel, the natural route of infection is by ingestion of OV as a contaminant of the food source (per os infection). In this report, we have examined the per os infectivity of mutant viruses lacking *p35*, *p94*, or both genes.

Viruses carrying deletions within *p35* (vP35del), *p94* (vP94del), or both *p35* and *p94* (vP3594del) were constructed (Fig. 1) by using previously published techniques (15). It was necessary to construct a new *p35* null mutant because both previously studied *p35* null mutants, vAcAnh and vP35Z (2, 3), are inappropriate for oral bioassays. vAcAnh is not isogenic to our wild-type (L-1) virus (13), and vP35Z has a second-site mutation affecting polyhedron formation (3). The infection of SF-21 cells with vP35del resulted in apoptosis (4) while the infection of TN-368 cells with this mutant resulted in an apparent wild-type infection. Thus, the apoptotic phenotype of vP35del was similar to those of vAcAnh and vP35Z (2, 3). The *p94* mutant, vP94del, exhibited an apparent wild-type phenotype in SF-21 and TN-368 cells while the double mutant,

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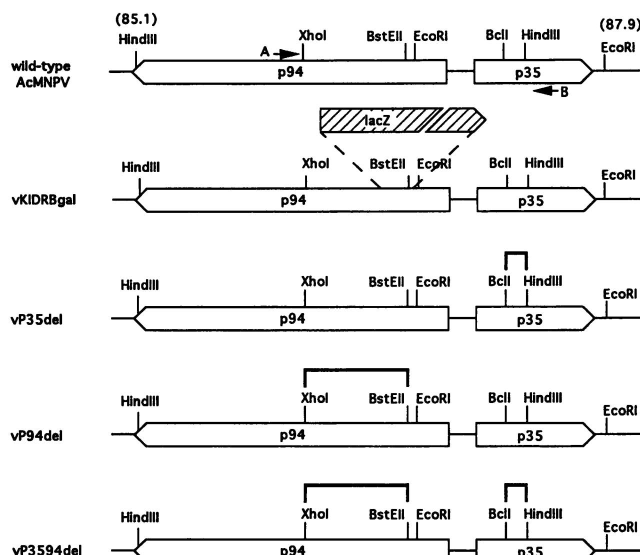


FIG. 1. The organization of the Ac*M*NPV genome in the region containing *p35* and *p94* and the construction of the mutant viruses used in this study. The region from 85.1 to 87.9 map units is shown, with the *p94* and *p35* open reading frames as open arrows. Dashed lines indicate the insertion of *lacZ* in vKIDRBgal while brackets indicate the regions deleted in vP35del, vP94del, and vP3594del. The break in the *lacZ* gene indicates that it is not drawn to scale. The deletion viruses vP35del and vP3594del were constructed by first cotransfecting SF-21 cells with vKIDRBgal DNA and plasmids containing the indicated deletions and then randomly screening plaques obtained from TN-368 cells by PCR (15) with oligonucleotide primers A and B (shown by arrowheads). vP94del was made by cotransfecting SF-21 cells with DNA from vKIDRBgal and a plasmid containing the indicated deletion and by screening for white plaques on SF-21 cells in the presence of a chromogenic substrate.

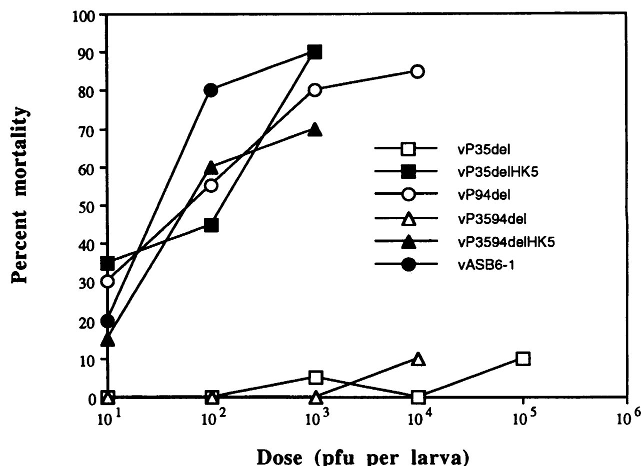


FIG. 2. Mortality in *S. frugiperda* larvae injected with the mutant virus vP35del, vP94del, or vP3594del, the control virus vP35delHK5 or vP3594delHK5, or vASB6-1, a *p35* mutant expressing the Cp-*iap* gene. Groups of 20 larvae which had molted into fifth instar less than 24 h earlier were injected with various doses of BV from each virus (as determined by plaque assay with TN-368 cells) diluted in tissue culture medium. No mortality was observed for mock-infected larvae injected with medium alone. The virus stocks used were retitered immediately following the injection experiment to ensure the titers had not changed significantly.

vP3594del, exhibited a phenotype similar to that of *p35* mutant viruses in cell culture, including the triggering of apoptosis in SF-21 cells (5). Control revertant viruses (vP35delHK5 and vP3594delHK5) were obtained by cotransfecting SF-21 cells with DNA from vP35del and vP3594del, respectively, and the lambda clone HK5, which contains wild-type Ac*M*NPV DNA approximately from 80 to 91 map units (16), and screening for a wild-type (occlusion-positive) plaque phenotype in SF-21 cells. The genomic structures of the viruses constructed were confirmed by restriction enzyme analysis and DNA hybridization (5).

In addition to its phenotype in cell culture, the infectivity of vP35del was assessed by determining the $LD_{50}$s obtained by hemocoelic injection of BVs into *S. frugiperda* larvae (Fig. 2). Like those of other *p35* null mutants (3, 5), the $LD_{50}$ of vP35del is so high that it is physically difficult to deliver a large enough dose to obtain a calculable $LD_{50}$ value. However, it is clear that the $LD_{50}$ of vP35del by this route was increased at least 1,000-fold compared with that of the control revertant virus vP35delHK5 (Fig. 2). The infectivity of the revertant viruses, vP35delHK5 and vP3594delHK5 (Fig. 2), was similar to that previously observed for wild-type Ac*M*NPV (3). Infectivity by hemocoelic injection did not appear to be significantly affected by the disruption of *p94*, since vP94del exhibited wild-type infectivity and the infectivity of vP3594del was similar to that of vP35del (Fig. 2).

The apoptosis-blocking function of *p35* in SF-21 cells can be replaced by an unrelated baculovirus gene, Cp-*iap*, obtained from *Cydia pomonella* granulosis virus (6). The expression of either *p35* or Cp-*iap* is sufficient to block apoptosis in SF-21 cells (4); presumably these two gene products act by different mechanisms, since they share no sequence similarity and the predicted product of Cp-*iap* contains sequence motifs characteristic of a DNA-binding protein, including a $C_3HC_4$ zinc finger (6). When larvae were injected with the budded form of vASB6-1, a recombinant virus which lacks *p35*, contains Cp-*iap*, and has a wild-type phenotype in SF-21 cells (1, 6), the 1,000-fold deficiency in infectivity associated with the lack of *p35* was restored to wild-type levels (Fig. 2). Thus, the decrease in BV infectivity resulting from the disruption of *p35* can be compensated for by the expression of the unrelated gene Cp-*iap*, which, like *p35*, is capable of blocking apoptosis in SF-21 cells. This result provides strong evidence that the reduction in infectivity seen for *p35* mutant BVs in *S. frugiperda* is due to apoptosis.

The effects of disrupting *p35*, *p94*, or both genes on per os infectivity were tested by allowing neonate larvae to feed on diet contaminated with various concentrations of OV obtained from *T. ni* larvae infected by injection with BV. In *S. frugiperda* larvae, a 25-fold increase in the concentration of vP35del OV was required for 50% lethality ($LC_{50}$) compared with that of the control virus vP35delHK5 (Table 1). However, the virus lacking both *p35* and *p94*, vP3594del, exhibited an $LC_{50}$ only twofold higher than that of the control virus vP3594delHK5; this twofold difference was not statistically significant as judged by probit analysis (7). The disruption of *p94* alone (vP94del) also had no significant effect on the $LC_{50}$ compared with that of wild-type Ac*M*NPV. The $LC_{50}$s of the control revertant viruses, vP35delHK5 or vP3594delHK5, were similar to that of wild-type Ac*M*NPV (L-1 strain) (13). The results obtained with *S. frugiperda* larvae were confirmed by two independent assays, and the differences observed were similar (within twofold) of those reported in Table 1.

The per os infectivity in *T. ni* larvae was unaffected by the

TABLE 1. Dose-mortality response of neonate *S. frugiperda* and *T. ni* larvae infected per os with viruses lacking *p35*, *p94*, or both genes[a]

| Host and virus | $LC_{50}$[b] (OV/ml) | 95% Fiducial limit[b] (OV/ml) | | Slope |
|---|---|---|---|---|
| | | Upper | Lower | |
| *S. frugiperda* | | | | |
| Wild type | $1.8 \times 10^6$ | $2.2 \times 10^6$ | $1.4 \times 10^6$ | 1.85 |
| vP35del | $3.3 \times 10^7$[c] | $1.4 \times 10^8$ | $1.6 \times 10^7$ | 0.86 |
| vP35delHK5 | $1.3 \times 10^6$ | $1.7 \times 10^6$ | $9.4 \times 10^5$ | 1.40 |
| vP94del | $7.3 \times 10^5$ | $1.6 \times 10^6$ | $2.3 \times 10^4$ | 1.19 |
| vP3594del | $2.3 \times 10^6$ | $3.1 \times 10^6$ | $1.6 \times 10^6$ | 1.18 |
| vP3594delHK5 | $1.2 \times 10^6$ | $1.6 \times 10^6$ | $9.4 \times 10^5$ | 1.74 |
| *T. ni* | | | | |
| Wild type | $2.9 \times 10^4$ | $3.3 \times 10^4$ | $2.5 \times 10^4$ | 2.96 |
| vP35del | $2.5 \times 10^4$ | $2.9 \times 10^4$ | $2.2 \times 10^4$ | 2.87 |
| vP35delHK5 | $1.9 \times 10^4$ | $2.2 \times 10^4$ | $1.6 \times 10^4$ | 3.13 |
| vP94del | $3.5 \times 10^4$ | $1.7 \times 10^5$ | $1.2 \times 10^4$ | 3.70 |
| vP3594del | $3.7 \times 10^4$ | $7.3 \times 10^5$ | $8.3 \times 10^3$ | 3.68 |
| vP3594delHK5 | $3.1 \times 10^4$ | $1.1 \times 10^5$ | $1.0 \times 10^4$ | 4.01 |

[a] Groups of 60 neonate larvae (less than 24 h old) were allowed to feed on diet contaminated with various concentrations of OV (five doses per virus) for 24 h, transferred to uncontaminated diet, and then observed for mortality.
[b] Values were calculated by probit analysis (7).
[c] Statistically different from appropriate control.

disruption of *p35*, *p94*, or both genes (Table 1). This result is consistent with our previous finding that the in vivo infectivity in *T. ni* larvae is unaffected by the disruption of *p35* when BV is injected into the hemocoel (3).

Although the disruption of *p35* resulted in a virus with decreased infectivity in *S. frugiperda*, the 50% survival time was not affected when any of the mutant viruses were used to infect either host species (Table 2). Thus, even though a higher $LC_{50}$ of vP35del OV was required in *S. frugiperda* (Table 1), the insects which became infected died at a rate which was similar to that of those infected by *p35*-containing viruses.

Insects of either species infected with vP35del or vP3594del either by injection of BV or by ingestion of OV also did not lose their basic morphological integrity (i.e., did not "melt"), as

TABLE 2. Time-mortality response of neonate *S. frugiperda* and *T. ni* larvae infected per os with viruses lacking *p35*, *p94*, or both genes[a]

| Host and virus | $ST_{50}$[b] (h) | 95% Fiducial limit[b] (h) | | Slope |
|---|---|---|---|---|
| | | Upper | Lower | |
| *S. frugiperda* | | | | |
| Wild type | 105 | 125 | 96 | 11.6 |
| vP35del | 105 | 116 | 94 | 9.3 |
| vP35delHK5 | 103 | 112 | 95 | 9.9 |
| vP94del | 101 | 109 | 94 | 12.9 |
| vP3594del | 93 | 101 | 85 | 10.5 |
| vP3594delHK5 | 105 | 113 | 98 | 12.7 |
| *T. ni* | | | | |
| Wild type | 79 | 84 | 75 | 11.0 |
| vP35del | 75 | 80 | 71 | 9.9 |
| vP35delHK5 | 78 | 82 | 73 | 10.3 |
| vP94del | 84 | 88 | 80 | 12.4 |
| vP3594del | 80 | 85 | 75 | 10.7 |
| vP3594delHK5 | 77 | 81 | 73 | 11.6 |

[a] The insects infected for the experiment in Table 1 were monitored every 12 to 24 h, and their times of death were recorded.
[b] Values were determined by probit analysis (7) at an approximate $LC_{50}$ in *S. frugiperda* and an approximately 95% lethal concentration in *T. ni*. $ST_{50}$, 50% survival time.

was previously observed with vAcAnh- and vP35Z-injected larvae (3). Insects infected with virus vP94del, vP35delHK5, or vP3594delHK5 exhibited normal melting. Interestingly, insects which died after the injection of vASB6-1 also did not melt. Although the basis for larval melting is not fully understood physiologically, it is likely that the inability of the virus to infect certain tissues, such as the muscular tissue of the body wall, affects the melting of the insect. If so, the inability of mutants to cause melting may be ascribed to the inability of those mutants to invade or replicate in key tissues affecting melting. Thus, *p35* may be required to block apoptosis in specific tissues of both species and *iap* is unable to compensate for *p35* in all tissues.

There are several possible explanations for the observation that infectivity is affected less by the disruption of *p35* when the virus is administered per os than by hemocoelic injection. The most unlikely of these is that the *p35* gene product may be required for the infectivity of BV but not OV in *S. frugiperda* in some way that does not involve apoptosis (e.g., virus attachment or uncoating). This explanation is unlikely because the unrelated apoptosis-inhibiting gene Cp-*iap* was able to restore BV infectivity (Fig. 2), strongly suggesting that the decreased infectivity by injection is due to apoptosis. Another relatively unlikely possibility is that the 25-fold increase in the $LC_{50}$ of OV associated with the disruption of *p35* may actually reflect a 1,000-fold difference in the infectivity of occluded virions in midgut cells, the site of primary infection by the oral route. The basis for this possibility resides in the fact that each occlusion body contains multiple virions. If one assumes that each larva which is fed an $LC_{50}$ dose of wild-type virus receives one infectious occlusion body and that each infectious occlusion body initiates multiple (e.g., 40) infectious foci in the midgut, then a 25-fold increase in the number of occlusion bodies required to initiate a lethal infection in a larva may actually represent a 1,000-fold increase ($25 \times 40$) in the number of infectious foci in the midgut. An additional assumption would be that any one of the foci initiated by wild-type virus in the midgut could lead to insect mortality; if this explanation is correct, then it would follow that only 1 in 1,000 foci initiated by *p35* mutant virus could lead to larval death. Establishing 40 infectious foci per occlusion body would also require that the virions liberated following occlusion body solubilization are highly efficient in initiating infectious foci once solubilization occurs at the appropriate point in the midgut. Although it seems unlikely that this explanation could be solely responsible for the relative differences in *p35* mutant BV and OV infectivities, it may contribute to the difference. If multiple foci per infectious occlusion body is the basis for the difference, the suppressive effects of the *p94* mutation in midgut cells become even more striking.

Additional possible explanations for the differences in the relative infectivities of *p35* mutant BVs and OVs involve tissue-specific differences in sensitivity to apoptosis. The tissues which are important in oral infection may be less sensitive to *p35* mutant-induced apoptosis than tissues which are important for infection by hemocoelic injection. For example, if midgut cells were 40-fold less sensitive to the induction of apoptosis than were hemocytes, which are probably the primary site of infection following BV injection, then one might expect a 25-fold difference in infectivity by the two routes of virus administration. The tissues involved in the pathogenesis of Ac*M*NPV by either route are not well understood. Data from oral-feeding studies suggest that midgut cells are the primary site of infection and that once they are infected, the secondary spread of infection occurs by cell-to-cell contact as well as by free BV (12). The types of cells reported to be

involved in the spread of infection following oral administration are mainly tracheal epithelial cells and hemocytes (12). Hemocytes become heavily infected following infection by either route and are likely to be the primary site of infection following hemocoelic injection.

Explanations based on tissue-specific sensitivity to apoptosis must also provide an explanation for the observations that (i) the 50% survival time is unaffected by the disruption of *p35* and (ii) the double *p35/p94* mutant is severely defective in BV infectivity but normal in OV infectivity as well as 50% survival time. These observations suggest that once infection is established, secondary infection proceeds with normal efficiency despite reduced BV infectivity in the hemocoel. Although contrary to present dogma, it is possible that the successful secondary spread of infection following oral infection does not require BV infection of hemocytes. Virus which has entered per os might still spread efficiently by other routes, such as cell-to-cell contact, or by BV infection of other cell types.

The ability of a deletion in *p94* to suppress the phenotype of a *p35* mutant, restoring per os infectivity, was unexpected. The suppression of apoptosis does not occur in SF-21 cells infected with the double mutant vP3594del (5), and the infectivity of the double mutant was similar to that of the *p35* mutant in larvae infected by injection (Fig. 2). Thus, whatever is the basis for the differences in *p35* mutant BV and OV infectivities, the suppressive effects of the *p94* mutation are specific to the route of infection, implicating tissue specificity in the apoptotic response.

On the basis of our current knowledge of this system, the most likely interpretation of the mutant phenotypes is that *p94* alters *S. frugiperda* midgut cells (and/or another cell type essential for per os infection) so that they are susceptible to apoptosis, thus requiring *p35* to block this response. This interpretation suggests that *p35* and *p94* have coevolved, a possibility which is consistent with their colocalization in the genome and with the observation that the related baculovirus *O. pseudotsugata* NPV lacks both *p35* and *p94* (10). It is not clear what advantage the expression of *p94* might have on Ac*M*NPV infections, but it is likely that the clue to *p94* function will come from studying the oral infectivities of *p94* deletion mutants in different insect species. We conclude that *p35* enhances the infectivity of both the budded and occluded forms of Ac*M*NPV in *S. frugiperda* larvae and that *p35* most likely acts by blocking apoptosis induced by the virus in a tissue-specific manner as well as species-specific manner. Virus-induced apoptosis in vivo, however, remains to be formally demonstrated.

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