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Autographa californica M nucleopolyhedrovirus early GP64 synthesis mitigates developmental resistance in orally infected noctuid hosts

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The unusual early synthesis of the *Autographa californica* M nucleopolyhedrovirus (AcMNPV) budded virus (BV) structural protein GP64 is an important virulence factor during oral infection of *Heliothis virescens* larvae. Considering the breadth of the AcMNPV host range, the importance of early GP64 synthesis in orally infected permissive hosts (*Trichoplusia ni* and *Spodoptera exigua*) from subfamilies other than that of *H. virescens* was assessed. An AcMNPV recombinant, having wild-type early and late GP64 synthesis, was compared with one in which only late GP64 synthesis occurred. Early GP64 synthesis was found to have more of an effect on virulence in orally inoculated *T. ni* than *S. exigua* and that virulence was dependent on two factors: the ability of the host to slough occlusion-derived virus (ODV)-infected midgut cells and the rapidity with which BV was transmitted to the tracheal cells. In both host species, insects inoculated orally with the control virus transmitted BV to tracheal cells hours before those inoculated with the *gp64* temporal mutant. Moreover, with early GP64 synthesis, the lag between the onset of viral gene expression in midgut and tracheal cells was only 3–4 h, supporting the conclusion that in these insects, the first systemic infections arose from ODV-derived nucleocapsids repackaged as BV. These results provide further empirical proof that early GP64 synthesis is a component of a unique and selectively advantageous baculovirus infection strategy for exploiting larval lepidopterans by counteracting developmental resistance.

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INTRODUCTION

Autographa californica M nucleopolyhedrovirus (AcMNPV) (*Baculoviridae*: *Nucleopolyhedrovirus*) is a promising microbial agent for the control of lepidopteran pests, in part because it can fatally infect the larvae of more than 30 species in 12 families, including significant crop pests in the Noctuidae, the most specious of the lepidopteran families (Adams & McClintock, 1991; Granados & Williams, 1986). Nucleopolyhedroviruses (NPVs) require two phenotypes to complete their infection cycles *in vivo*. The first form, occlusion-derived virus (ODV), is embedded in a crystalline matrix of polyhedrin and initiates primary infection in the host's midgut epithelium following ingestion and dissolution of the viral occlusions. The second form, budded virus (BV), subsequently disseminates the infection throughout the host until the insect succumbs and liquefies, releasing millions of viral occlusions that can then transmit infection to new hosts.

While AcMNPV ODV and BV are genetically identical and share subsets of structural polypeptides, some polypeptides are distinct. These structural differences undoubtedly relate to the fact that the two viral phenotypes target different types of cells, infect under very different physiological conditions and use different mechanisms to penetrate their host cells (Braunagel & Summers, 1994; Volkman, 1983, 1997). GP64 is the major BV envelope glycoprotein of the Group I NPVs and is acquired when BV particles bud through the plasma membrane (Blissard & Rohrmann, 1989; Volkman *et al.*, 1984; Whitford *et al.*, 1989). GP64 is essential for AcMNPV BV infectivity (Monsma *et al.*, 1996) and has a highly unusual temporal pattern of expression, being synthesized during both early and late phases of infection. This expression pattern is unlike that of all other AcMNPV structural proteins (and indeed viral structural proteins in general), which are only synthesized during the late phase of infection (Blissard & Wenz, 1992; Hefferon *et al.*, 1999; Monsma *et al.*, 1996; Oomens & Blissard, 1999;

Volkman, 1986). While GP64 is essential for infection, elimination of its early synthesis impacts neither the timing nor the amount of BV produced *in vitro* and has no effect on BV virulence *in vivo* (Washburn *et al.*, 2003a). Interestingly, while GP64 is not a component of AcMNPV ODV, its early synthesis is a significant virulence factor during oral infection of the permissive host, *Heliothis virescens* (Washburn *et al.*, 2003a).

The key to understanding this apparent paradox lies in a second trait shared by AcMNPV and other MNPVs: multiple nucleocapsids within a single ODV envelope (the 'M' designates 'Multiple'). The major consequence of this novel phenotype is that during primary infection multiple nucleocapsids from an individual ODV enter the same midgut cell. Notably, wild-type AcMNPV ODV particles containing multiple nucleocapsids are much more virulent *per os* in *Trichoplusia ni* larvae than ODV particles containing a single nucleocapsid (Washburn *et al.*, 1999). Moreover, studies on AcMNPV pathogenesis have revealed that the onset of BV infection of secondary target cells in several host species occurs much too rapidly to be explained by *de novo* virus replication (Engelhard *et al.*, 1994; Flipsen *et al.*, 1995; Granados & Williams, 1986; Washburn *et al.*, 1995, 1998, 2000, 2003a, b). These studies have provided compelling evidence that AcMNPV (and, by extension, other Group I MNPVs) has evolved a highly unusual infection strategy for exploiting lepidopteran larvae. Specifically, it appears that only a fraction of the nucleocapsids from an ODV particle enters the midgut cell nucleus and uncoats during primary infection, enabling expression of *gp64* as an early gene product. At the same time, a separate subpopulation of incoming nucleocapsids migrates to the basal plasma membrane and is repackaged as BV with a membrane containing newly synthesized GP64. This apparently allows the midgut cells infected with AcMNPV ODV to transmit secondary infections to target cells within the host's haemocoel hours before *de novo* synthesis of BV; this would explain the remarkably rapid onset of secondary infections. Such an infection strategy would give the virus a strong selective advantage because it would obviate one of the principal host defences against baculovirus infection, sloughing ODV-infected midgut cells.

We previously investigated the biological significance of the unusual biphasic mode of GP64 synthesis in *H. virescens* larvae by comparing the virulence and pathogenesis of AcMNPVs that express *gp64* both early and late or only during the late phase of infection. The results of this study were consistent with the nucleocapsid repackaging hypothesis detailed above, as they showed that in *H. virescens* early GP64 synthesis increased oral virulence and accelerated the onset of ultimately fatal secondary infections within the host's tracheal system (Washburn *et al.*, 2003a). Here we report results from similar studies in which we evaluated the effects of early GP64 synthesis on AcMNPV virulence and pathogenesis in two additional permissive hosts, *Spodoptera exigua* and *T. ni*. These insects are significant agricultural pests, and each is from a different subfamily of the Noctuidae (*H. virescens* – Heliothinae; *T. ni* – Plusinae; *S. exigua* – Amphipyrinae). While early GP64 synthesis increased ODV virulence during oral infection of newly moulted fourth instar *T. ni* larvae, it had no impact on ODV virulence in *S. exigua* larvae. Furthermore, the absence of any measurable effect of early GP64 synthesis on AcMNPV virulence in this cohort of *S. exigua* was correlated with two phenomena: (i) remarkably fast and efficient primary infection and (ii) a slow rate of sloughing ODV-infected midgut cells. In contrast, when inoculated 16 h after moulting, early GP64 synthesis was a significant virulence factor in larvae of both species, apparently counteracting the increasing developmental resistance characteristic of older hosts (Engelhard & Volkman, 1995). Finally, the virus that expressed *gp64* in an early–late, wild-type manner established secondary infections in both hosts hours earlier than the virus that only expressed *gp64* during the late phase of infection. These results provide further experimental evidence that early GP64 synthesis is a component of a unique and highly adaptive baculovirus infection strategy for counteracting developmental resistance mechanisms in their insect hosts.

METHODS

Virus preparation and quantification. Construction of two AcMNPV recombinants, AcCtlNt-64HB and AcLate21/20-64HB, used in this study has been described previously (Washburn *et al.*, 2003a). AcCtlNt-64HB (hereafter AcCtlNt) has wild-type *gp64* expression (early and late), and AcLate21/20-64HB (hereafter Ac21/20) expresses *gp64* only during the late phase of infection, coincident with the expression of other viral structural genes. Both viruses contained the *Escherichia coli* β-galactosidase gene (*lacZ*) under the control of a *Drosophila* heat shock promoter (*hsp70*), which allowed detection of infected cells by the presence of the blue reporter signal (Engelhard *et al.*, 1994). Occlusions and BV of both viruses were harvested at 96 h p.i. from cultured Sf-9 cells. Occlusions were further purified by sucrose gradient centrifugation, suspended in a neutrally buoyant solution of glycerin and water (3 : 2, v/v), quantified using a haemocytometer and stored at 4 °C until used. BV was titrated by plaque assay on Sf-9 cells (Volkman & Goldsmith, 1981) and held at 4 °C until used. A third recombinant virus, vAc^{64z}, which was kindly provided by Gary Blissard (Boyce Thompson Institute at Cornell University, Tower Road, Ithaca, NY, USA) also carries the *hsp70*/*lacZ* marker but is unable to synthesize any GP64 (Monsma *et al.*, 1996). vAc^{64z} was used to quantify the retention of ODV-infected midgut cells during moulting.

Insects. Eggs of *S. exigua* were provided by the USDA-ARS Western Cotton Research Laboratory, and *T. ni* eggs were purchased from Benzon Research (Carlisle, PA, USA). Larvae were reared on synthetic Stoneville diet at 22 or 28 ± 3 °C under constant light through the third instar; under these conditions, both species had five larval instars. Quiescent, late third instar larvae that were preparing to moult were sequestered and observed carefully to determine the exact time of moulting for each insect. In some cases, third instar larvae were held at 7 °C from several hours to overnight in order to regulate their developmental rates and to make large numbers of test insects of the same age available for experiments (Washburn *et al.*, 1995).

Bioassays and time-course experiments. To establish oral dose–mortality relationships for AcCtlNt and Ac21/20, bioassays were done using two developmental cohorts of *S. exigua* and *T. ni*. These cohorts were newly moulted and 16 h post-moult fourth instar larvae, hereafter designated 4^0 and 4^{16}, respectively. Individual larvae were inoculated using a Burkhard microinjector with a 1 cm^3 tuberculin syringe fitted with a 32 gauge blunt-tipped needle. The needle was inserted through the mouth and into the midgut lumen where the occlusion suspension (0·25 to 1·0 µl in volume) was discharged. Virulence of the BV phenotypes was determined by intrahaemocoelic inoculation of cohorts of 24 h post-moult fourth instar (4^{24}) larvae. For these assays, a sharp-tipped 32 gauge needle was inserted through one of the prolegs, and the viral suspension (1 µl volume) was discharged into the larval haemocoel (Engelhard & Volkman, 1995; Engelhard *et al.*, 1994; Washburn *et al.*, 1995). Between 26 and 32 insects were used for each assay. After inoculation, test larvae were maintained individually in 25 ml plastic cups containing diet *ad libitum* in a growth chamber at 28 ± 2 °C until pupation or death from polyhedrosis disease. Viral death was confirmed by microscopic examination (400×) of cadaver tissues for occlusions. Dose–mortality relationships for each virus–host treatment were evaluated with linear regression using the least squares method.

To investigate the pathogenesis of AcCtlNt and Ac21/20, a series of time-course experiments was carried out using 4^0 larvae. For these assays, we utilized dosages (determined from bioassays described above) that yielded final mortalities of ~90 %. *S. exigua* were inoculated with 10 occlusions of either recombinant, and *T. ni* were inoculated with 21 or 30 occlusions of either AcCtlNt or Ac21/20, respectively. At various times during the first 24 h post-inoculation (p.i.), cohorts of between 26 and 32 larvae of each species from the two viral treatments were dissected, and their midguts and associated tissues removed. These tissues were processed to detect the presence of β-galactosidase and examined using stereo (10–50×) and/or compound microscopy (100–480×) in order to quantify infection foci and identify infected cell types (Engelhard *et al.*, 1994; Washburn *et al.*, 1995, 2001, 2003a). For each host species, an additional cohort of 32 insects was inoculated orally with AcCtlNt or Ac21/20 occlusions to confirm that each dosage was an LD_{90}.

In a separate experiment, we quantified retention of ODV-infected midgut cells through the moult to the fifth instar using vAc^{64z}. This recombinant cannot synthesize GP64 and can only establish primary (midgut) infections following oral inoculation with occlusions (Monsma *et al.*, 1996). A dosage of 175 occlusions (i.e. sufficient to generate large numbers of primary foci) was used to infect cohorts of 4^0 and 4^{16} *S. exigua* and *T. ni* larvae for time-course experiments similar to those described above.

RESULTS

The dose–mortality relationships for fourth instar larvae of *S. exigua* and *T. ni* inoculated orally with occlusions of either AcCtlNt (control) or Ac21/20 (expresses only late GP64) are presented in Fig. 1. Both developmental cohorts of fourth instar *S. exigua* were more susceptible to fatal infection by AcCtlNt and Ac21/20 than the corresponding cohorts of *T. ni*. Even so, the 4^0-inoculated larvae of both species were highly susceptible; a single AcCtlNt occlusion generated 21·9 and 12·5 % larval mortality in *S. exigua* and *T. ni*, respectively (Fig. 1A). Unexpectedly, newly moulted *S. exigua* had virtually identical susceptibility to AcCtlNt and Ac21/20 (LD_{50}s of 4 occlusions; LD_{90}s of 10 and 12 occlusions for AcCtlNt and Ac21/20, respectively; Fig. 1A, B), demonstrating that for this developmental stage early GP64 synthesis was inconsequential to oral virulence. In contrast, the AcCtlNt LD_{50} for 4^{16} *S. exigua* was 8 occlusions, demonstrating that even in this extremely susceptible host, a twofold increase in wild-type viral inoculum was required to overcome the developmental resistance acquired during the first 16 h after moulting. For Ac21/20, however, an 8·75-fold increase in inoculum (35 occlusions) was needed to achieve an LD_{50} in 4^{16} *S. exigua*. Hence, early *gp64* expression provided a 4·4-fold increase in the ability of AcMNPV to overcome the developmental resistance acquired by the older larvae.

In *T. ni*, early *gp64* expression had an even greater impact on virulence. First, unlike the 4^0 *S. exigua*, in which early GP64 synthesis was inconsequential, 1·8-fold more Ac21/20 occlusions were required for an LD_{50} in *T. ni*, as compared to AcCtlNt (11 and 6 occlusions, respectively). This shows that following oral inoculation of newly moulted larvae, early GP64 synthesis reduced by almost half the amount of inoculum needed for an LD_{50} and therefore significantly increased virulence. In 4^{16} *T. ni*, the cost of not expressing *gp64* during the early phase of infection was even greater, as 9·4-fold more inoculum was required for an LD_{50} in this cohort compared to 4^{16} insects inoculated with AcCtlNt (113 vs 12 occlusions). These results showed that *T. ni* has greater developmental resistance to AcMNPV infection than *S. exigua*, and that the ability of early GP64 synthesis to enhance virulence was proportional to the degree of developmental resistance (Washburn *et al.*, 2003a).

The developmental resistance acquired by *T. ni* during the fourth instar was shown previously to result from the reduced ability of AcMNPV to establish and/or maintain primary infections within the midgut (Engelhard & Volkman, 1995; Washburn *et al.*, 1998). To determine if the basis for developmental resistance was the same in *S. exigua*, we orally inoculated 4^0 and 4^{16} larvae with 10 occlusions of AcCtlNt ($n = 28$ for each treatment) and used β-galactosidase as a reporter to quantify viral foci at 12 h p.i. Relative to insects inoculated immediately after moulting, 57 % fewer foci were observed in 4^{16} larvae [$4^0 = 20{\cdot}3 \pm 4{\cdot}7$ (mean ± 1 SE) foci; $4^{16} = 8{\cdot}8 \pm 2{\cdot}9$ foci], which was consistent with the fact that the inoculum had to be doubled to achieve an LD_{50}. Moreover, the decrease in foci number suggested that *S. exigua* and *T. ni* shared a similar physiological basis for developmental resistance to AcMNPV.

In contrast to the differences in oral virulence between AcCtlNt and Ac21/20 shown in Fig. 1, these viruses were equally and highly virulent when the midgut was bypassed by injecting BV directly into the host's haemocoel. Following injection into 4^{24} *S. exigua*, 0·13 p.f.u. of AcCtlNt and Ac21/20 yielded final mortalities of 19 and 11 %, respectively, and dosages of 3·3 p.f.u. yielded final mortalities of 81 and 82 %, respectively. Both viruses provided similar results in *T. ni*. For example, following inoculation of 4^{24} *T. ni* larvae with 3·3 p.f.u. of AcCtlNt, 83 % of the

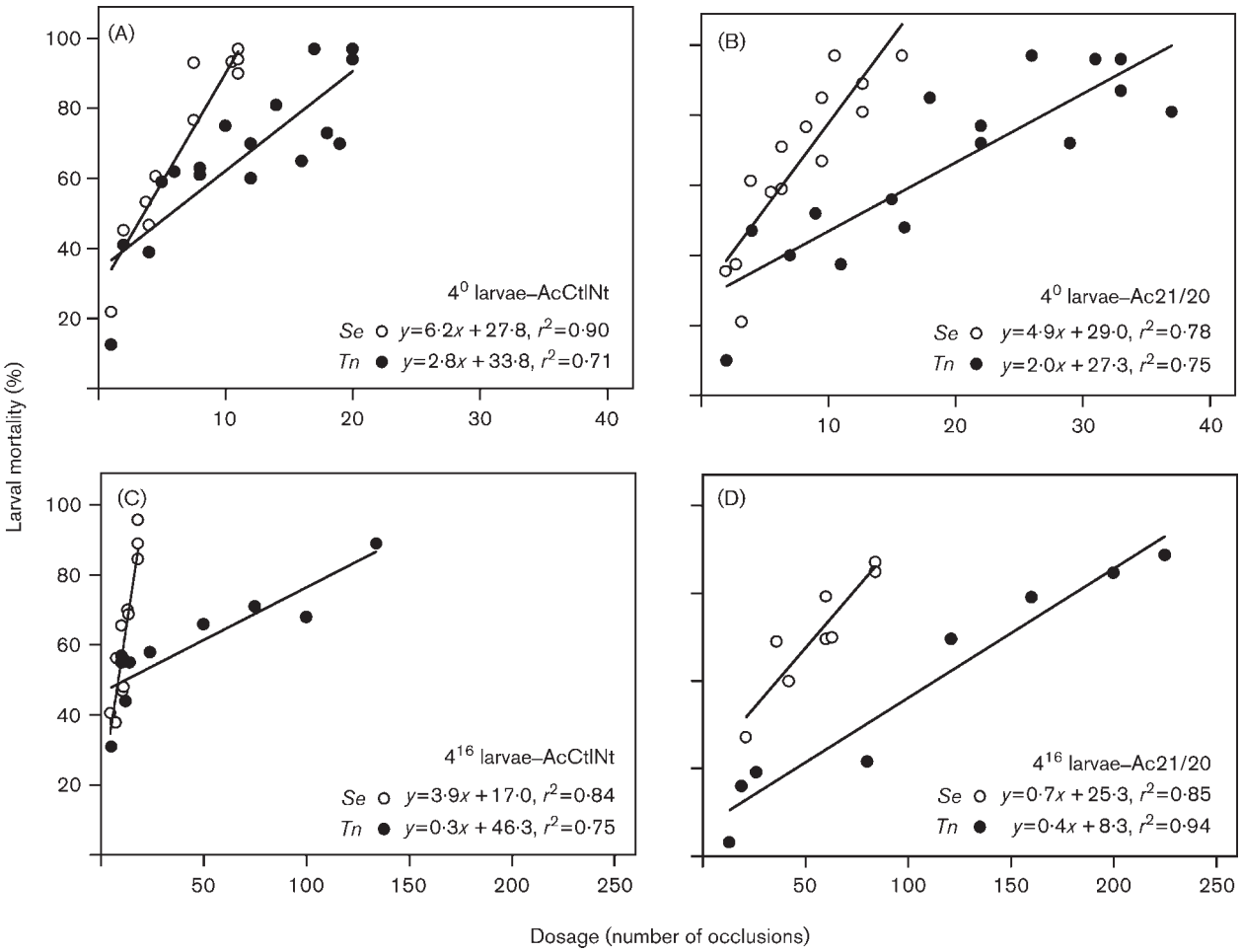

Fig. 1. Dose–mortality relationships for *S. exigua* (*Se*) and *T. ni* (*Tn*) larvae inoculated orally as newly moulted fourth instars (A and B, respectively) or 16 h after the moult (C and D, respectively) with occlusions of either AcCtlNt or Ac21/20. Each point represents the percentage of a cohort containing between 22 and 32 insects that died from baculovirus infection while in the larval stage.

larvae succumbed to polyhedrosis disease. These findings demonstrated that early GP64 synthesis was only important for virulence via the natural route of infection (*per os*). The fact that both hosts were equally susceptible to injection of minute quantities of BV underscored the importance for AcMNPV to establish even a single foothold of infection within the haemocoel.

The results of the time-course experiments in which AcCtlNt and Ac21/20 pathogenesis in *S. exigua* and *T. ni* larvae was monitored with the *lacZ* reporter are shown in Figs 2–7. As expected, differences in the temporal pattern of *gp64* expression did not affect the onset of viral gene expression in ODV-infected midgut cells of either host (Fig. 2A, B; Washburn *et al.*, 2003a). With the dosages used in these experiments (LD_{90}), β-galactosidase, which is indicative of early viral gene expression, was detected at the same times after infection of each species by either virus (4 h p.i. in *S. exigua* and 6 h p.i. in *T. ni*; Figs 2A, B). Consistent with the earlier onset of *lacZ* expression in *S. exigua*, the rates of primary infection by both viruses were also much greater in *S. exigua* than in *T. ni*. Remarkably, with both viruses, the proportion of *S. exigua* larvae positive for LacZ was predictive of the final mortality as early as 8 to 12 h p.i. By contrast, the time point predictive of final mortality for AcCtlNt-infected *T. ni* was 24 h p.i. (Fig. 2A), 12 to 16 h later. Moreover, only 75 % of the cohort inoculated with Ac21/20 was β-galactosidase-positive at 24 h p.i. (Fig. 2B), indicating that 15 % of the larvae that would ultimately die were not yet expressing *lacZ*. These results suggested that the severity of developmental resistance was correlated with the rate at which the virus was able to establish gene expression in midgut cells.

The temporal pattern of viral focus formation was consistent with the greater virulence of both viruses in *S. exigua* than in *T. ni*. Despite being inoculated with fewer occlusions, both viruses produced more numerous and persistent primary foci in *S. exigua* than in *T. ni* (Fig. 3A, B). After controlling for dosage, each AcCtlNt occlusion produced

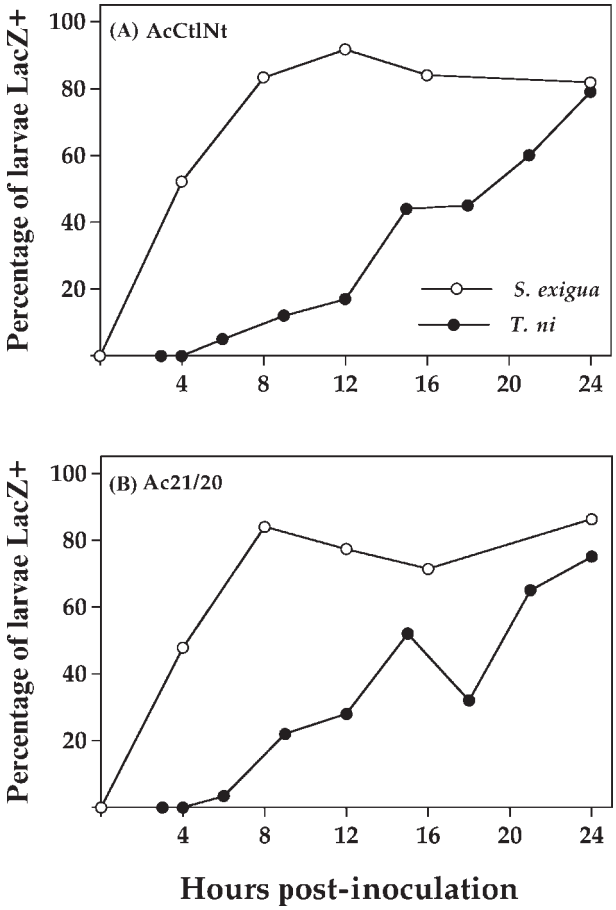

Fig. 2. Percentages of *S. exigua* and *T. ni* larvae positive for LacZ at various hours after inoculation as newly moulted fourth instars with either AcCtlNt (A) or Ac21/20 (B). *S. exigua* were inoculated with 10 occlusions of either AcCtlNt or Ac21/20, and *T. ni* larvae were inoculated with 21 or 30 occlusions, respectively, of either AcCtlNt or Ac21/20. All doses yielded a final larval mortality level of ~90% (Fig. 1). Each point represents the proportion of LacZ-positive larvae from a cohort of between 26 and 32 larvae.

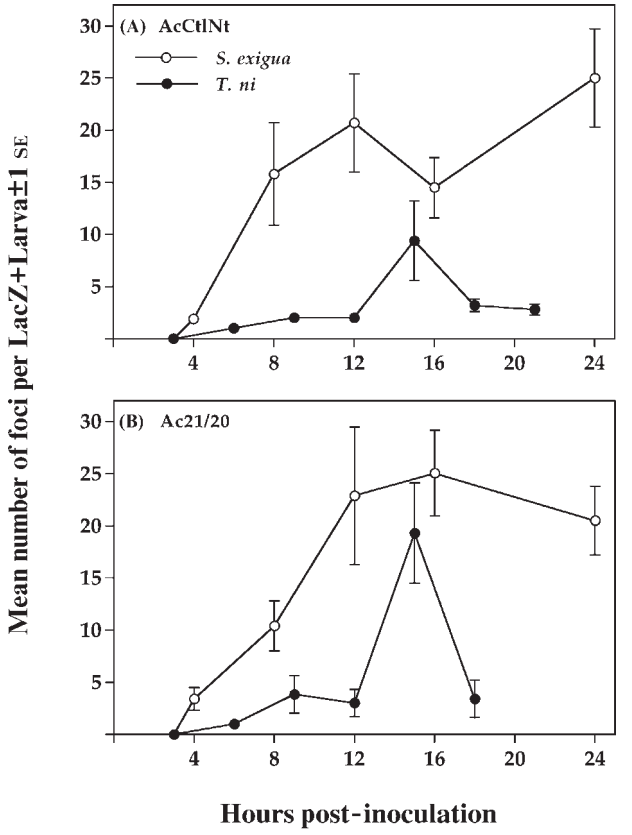

Fig. 3. Mean numbers of viral foci (±1 SE) in *S. exigua* and *T. ni* larvae at various hours after inoculation as newly moulted fourth instars with either AcCtlNt (A) or Ac21/20 (B). *S. exigua* larvae were inoculated with 10 occlusions of either AcCtlNt or Ac21/20, and *T. ni* larvae were inoculated with 21 or 30 occlusions, respectively, of either AcCtlNt or Ac21/20. Each point represents the mean foci numbers in LacZ-positive larvae from a cohort of between 26 and 32 larvae. Due to the confluence of viral plaques, Ac21/20 foci for *T. ni* sampled at 24 h p.i. could not be quantified.

a mean of 2·5 foci in *S. exigua*, as compared to only 0·4 foci in *T. ni*, and the ratio of focus formation for Ac21/20 was 2·5 : 0·6. Thus, these viruses produced six- and four-fold more foci in *S. exigua* than in *T. ni*, respectively. Moreover, foci numbers rose more slowly in *T. ni* larvae, peaked at 15 h p.i. and then abruptly declined (Fig. 3A, B; Fig. 4, Washburn *et al.*, 1999). At 18 h p.i., the numbers of foci observed in AcCtlNt and Ac21/20-infected *T. ni* larvae were reduced by 66 and 83% as compared to 15 h p.i., suggesting that many ODV-infected midgut cells had been sloughed. The observation that more Ac21/20 than AcCtlNt foci were required to achieve an LD_{90} in *T. ni* showed that the ODV of the latter was more efficient at establishing systemic infections.

During the early stages of viral pathogenesis, the primary midgut cellular targets of AcCtlNt and Ac21/20 in both *S. exigua* and *T. ni* were mature columnar epithelial cells. Similarly, the second cell type expressing *lacZ* in both hosts was tracheolar cells servicing ODV-infected midgut cells; the proximity of primary and immediate secondary targets indicated that these tracheolar cells were infected directly by BV generated from the ODV-infected midgut cells. Infection of tracheolar cells signalled the onset of irreversible fatal systemic infections, and for all four virus–host combinations, β-galactosidase signals within the tracheal epidermis rose linearly over time and at virtually identical rates [i.e. regression coefficients (slopes) of 3·8–4·1, Fig. 4A, B]. While the BVs of both viruses established secondary infection at similar rates, the onset by Ac21/20 was delayed by several hours relative to AcCtlNt. Using the linear equations correlating the prevalence of tracheal cell-containing viral foci with hours p.i. (Fig. 4A, B), the predicted times for the onset of tracheal infection of *S. exigua* larvae by AcCtlNt and Ac21/20 BV were 4·2 and 9·6 h p.i.,

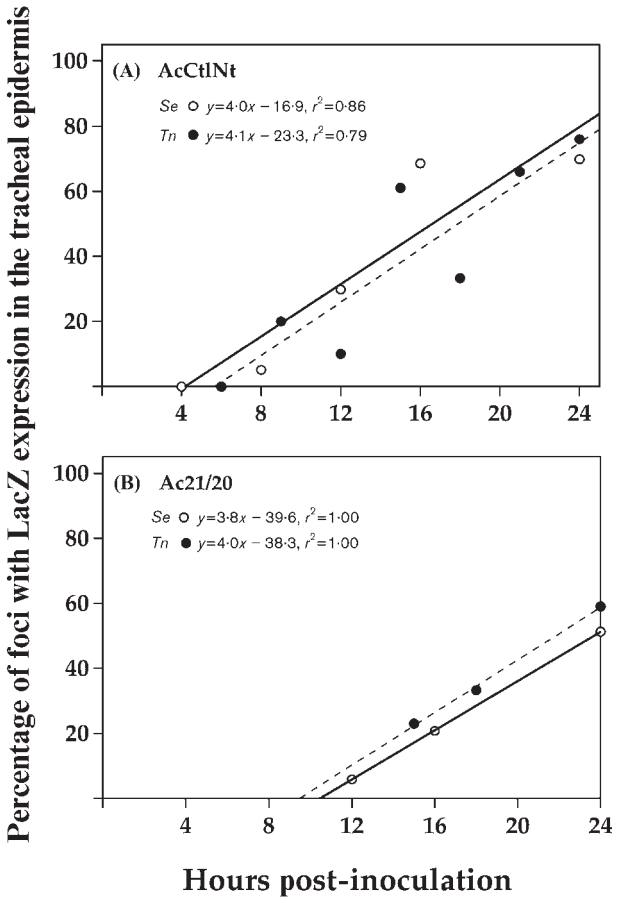

Fig. 4. Percentages of AcCtlNt (A) and Ac21/20 (B) foci with one or more LacZ-positive tracheal cells in *S. exigua* and *T. ni* at various times after inoculation as newly moulted fourth instars. *S. exigua* larvae were inoculated with 10 occlusions of either AcCtlNt or Ac21/20, and *T. ni* larvae were inoculated with 21 or 30 occlusions, respectively, of either AcCtlNt or Ac21/20. Each point represents mean foci numbers in LacZ-positive larvae from a cohort of between 26 and 32 larvae. Regression lines for *S. exigua* (*Se*) are solid, and those for *T. ni* (*Tn*) are dashed.

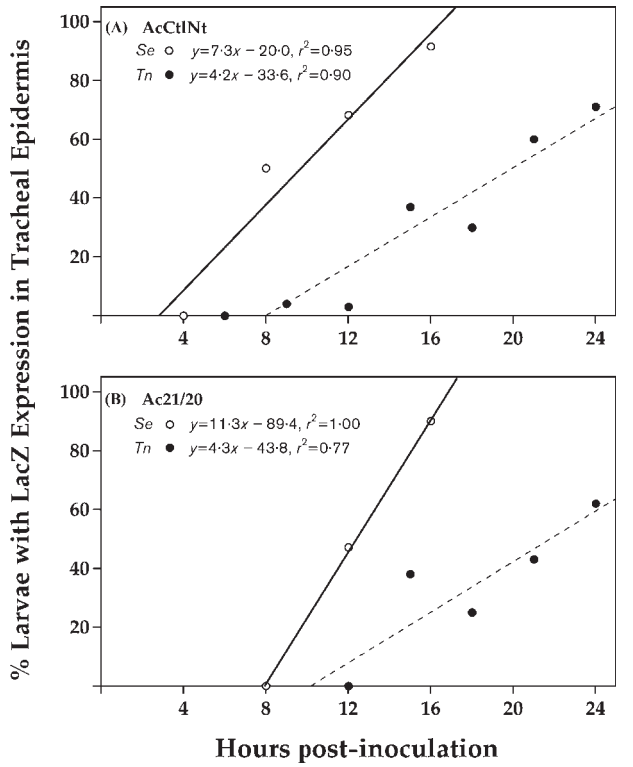

Fig. 5. Percentages of *S. exigua* and *T. ni* larvae with *LacZ* expression in the tracheal epidermis at various times after inoculation as newly moulted fourth instars with either AcNtlCt (A) or Ac21/20 (B). *S. exigua* larvae were inoculated with 10 occlusions of either AcCtlNt or Ac21/20, and *T. ni* larvae were inoculated with 21 or 30 occlusions, respectively, of either AcCtlNt or Ac21/20. Each point represents the proportion of larvae from a cohort of between 26 and 32 larvae. Regression lines for *S. exigua* (*Se*) are solid, and those for *T. ni* (*Tn*) are dashed.

respectively. In *T. ni* larvae the times predicted for the onset of tracheal infections were 5·7 and 10·4 h p.i. Thus, early GP64 synthesis accelerated establishment of systemic infection by 5·4 h in *S. exigua* and by 4·7 h in *T. ni*.

Due to the extreme sensitivity of these permissive hosts to the presence of BV within the haemocoel, the rates at which primary midgut foci established systemic infection, within even a single tracheal cell, accurately reflected the rates at which mortal infections were established within larval cohorts. Because the ODVs of both recombinants infected midgut cells of *S. exigua* more quickly and in greater numbers than in *T. ni* (Figs 2, 3), the proportions of *S. exigua* systemically infected by AcCtlNt and Ac21/20 rose much more rapidly than in the corresponding *T. ni* cohorts (Fig. 5A, B). This further explains why the percentages of β-galactosidase-positive *S. exigua* in both viral treatments were predictive of the final mortality many hours before *T. ni*.

In addition to temporal changes in foci numbers (Fig. 3A, B), the cellular composition of AcCtlNt and Ac21/20 foci provided further evidence that *T. ni* larvae sloughed infected midgut cells more frequently than *S. exigua*. During the early stages of AcMNPV pathogenesis *in vivo*, three kinds of viral foci can be distinguished: (i) midgut restricted, in which BV transmission to secondary targets has not occurred (Fig. 6A); (ii) midgut and tracheal, in which BV has been transmitted and the primary target remains (Fig. 6B); and (iii) tracheal, in which BV transmission has occurred and the primary target has been sloughed (Fig. 6D, C) (Washburn *et al.*, 1999, 2001, 2003a, b). Because tracheal epidermal cells are not exposed to the midgut lumen, they cannot be infected directly by ODV. Therefore, analysis of foci restricted to the tracheal epidermis provides a way to measure the rates at which infected larvae sloughed ODV-infected midgut cells. In our experiments with all four

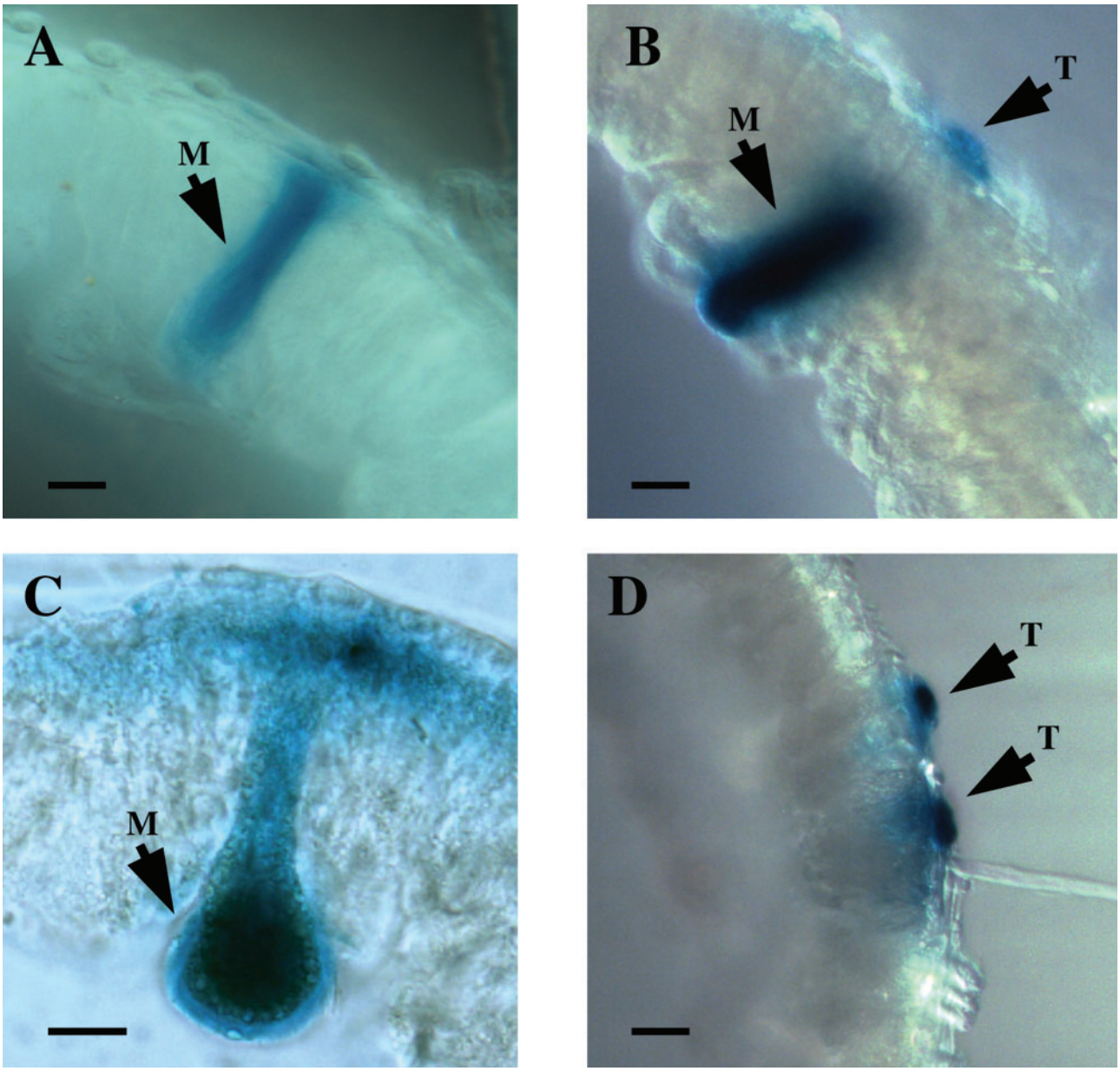

Fig. 6. Cellular composition of viral foci during early pathogenesis of AcCtlNt in *S. exigua* orally inoculated as newly moulted fourth instars. (A) Viral focus consisting of a single LacZ-positive columnar cell (M), the primary cellular target of ODV at ~18 h p.i. (B) Multicellular viral focus consisting of a LacZ-positive, ODV-infected columnar cell (M) and a BV-infected tracheolar cell (T) at ~18 h p.i. Tracheolar cells are the immediate targets of BV produced by ODV-infected midgut cells. (C) A LacZ-positive columnar cell (M) in the process of being sloughed from the midgut epithelium. Note the rounded apical cell body and the constricted cytoplasmic strand anchoring the cell to the midgut basement membrane. The image was captured from a newly moulted fifth instar insect ~44 h after inoculation. (D) Multicellular viral focus restricted to the tracheal epidermis and consisting of two LacZ-positive tracheolar cells (T) at ~24 h p.i. The underlying ODV-infected columnar cell presumably has been sloughed from the midgut epithelium. The bar in each panel is equal to 40 μm.

host–virus combinations, the temporal increases in the proportions of such foci were well described by linear models (Fig. 7A, B). For the two larval cohorts in each viral treatment, the ratio of the regression coefficients provided an estimate for the relative rates at which the primary midgut cellular targets infected by each virus were shed by *S. exigua* and *T. ni* larvae. These calculations indicated that *T. ni* larvae sloughed midgut cells infected by the ODVs of AcCtlNt and Ac21/20 at rates that were 4·8 and 14·9 times greater than *S. exigua*, respectively (Fig. 7A, B).

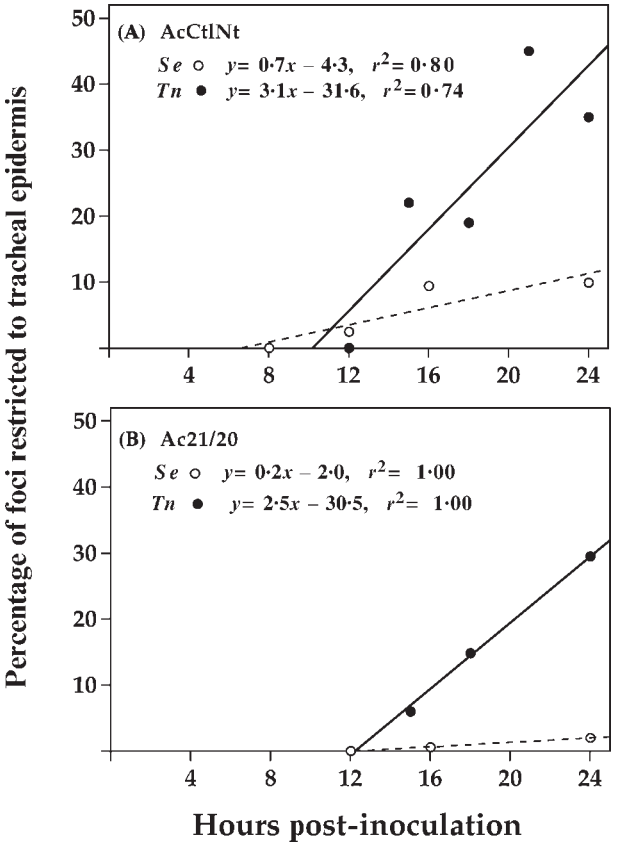

Fig. 7. Percentages of AcCtlNt (A) and Ac21/20 (B) viral foci restricted to the tracheal epidermis of *S. exigua* and *T. ni* larvae at various times after inoculation. *S. exigua* larvae were inoculated with 10 occlusions of either AcCtlNt or Ac21/20, and *T. ni* larvae were inoculated with 21 or 30 occlusions, respectively, of either AcCtlNt or Ac21/20. Each point represents the proportion of larvae from a cohort of between 26 and 32 larvae. Regression lines for *S. exigua* (*Se*) are solid, and those for *T. ni* (*Tn*) are dashed.

Notably, in our time-course experiments, some fifth instar *S. exigua* orally inoculated during the fourth instar were found to harbour β-galactosidase-positive midgut cells, implying that ODV-infected primary cellular targets in this host could be retained through the moult (Fig. 6C). This phenomenon was not observed in previous studies of AcMNPV-*hsp70*/*lacZ* pathogenesis in *T. ni* and *H. virescens* larvae because these species clear their midgut tissues of all ODV-infected cells during the first moult after inoculation (Washburn *et al.*, 1995). To determine if the midgut cell infections in fifth instar *S. exigua* could have resulted from BV transmission from infected tracheolar cells, we inoculated 255 p.f.u. of AcCtlNt BV intrahaemocoelically into feeding fourth instar larvae ($n=6$), killed the larvae at 24 h p.i., and examined their tissues for *lacZ* signals. While numerous β-galactosidase-positive cells were found within the tracheal epidermis on the distal side of the midgut basal lamina, no β-galactosidase-positive midgut epithelial cells were found, suggesting that midgut cells were not infected by BV from the haemocoel.

To quantify retention rates of ODV-infected midgut cells in *S. exigua* during larval moulting, another time-course experiment was conducted in which we orally inoculated 4^0 and 4^{16} *S. exigua* with 175 vAc^{64z} occlusions. This virus cannot synthesize GP64, and following oral inoculation of host larvae, only midgut infections ensue, facilitating rapid and accurate quantification of primary foci (Monsma *et al.*, 1996). Larvae in each *S. exigua* cohort were divided into two groups, dissected and examined for *lacZ* signals in midgut cells after reaching either late fourth or early fifth instar stages (4^0-inoculated – 40 and 48 h p.i.; 4^{16}-inoculated – 24 and 32 h p.i., respectively). The 4^0 larvae had $50{\cdot}5 \pm 7{\cdot}8$ and $13{\cdot}4 \pm 3{\cdot}1$ β-galactosidase-positive midgut cells in the fourth and fifth instar cohorts, respectively, and the 4^{16} larvae had $56{\cdot}5 \pm 11{\cdot}2$ and $43{\cdot}2 \pm 6{\cdot}4$ β-galactosidase-positive cells, respectively. Thus, 26 and 77 % of the ODV-infected primary foci produced during infection of 4^0 and 4^{16} *S. exigua* larvae and still present by the end of the fourth instar were retained through the moult to the fifth instar. When we repeated this experiment with the same fourth instar developmental cohorts of *T. ni*, no midgut cells infected by vAc^{64z} were detected in any fifth instar larva.

DISCUSSION

The results of the experiments reported here demonstrated that early expression of the AcMNPV BV structural protein GP64 was an important virulence factor during oral, but not intrahaemocoelic, infection of fourth instar larvae of the noctuids *T. ni* and *S. exigua*. The effect of early GP64 synthesis on AcMNPV virulence, however, varied significantly with species and developmental cohort. Early GP64 synthesis did not measurably enhance ODV virulence for newly moulted *S. exigua* larvae, whereas it strongly enhanced virulence for *T. ni* inoculated 16 h after moulting (Fig. 1). Overall, *S. exigua* larvae were more susceptible than *T. ni* to mortal infection by the ODVs expressing *gp64* in wild-type fashion (early–late) or only during the late phase of infection. The extreme oral susceptibility of *S. exigua* was attributed to three separate, but linked, phenomena: (i) the establishment of more foci per occlusion; (ii) the rapid rate at which primary cellular targets were infected; and (iii) the inability of larvae to shed ODV-infected midgut cells. Indeed, the sloughing response of fourth instar *S. exigua* was so weak that significant numbers of ODV-infected midgut cells were retained for as long as 48 h p.i., and in some cases even after larvae had moulted to the fifth and final larval instar.

The earliest detectable expression of *lacZ* in *S. exigua* midgut cells was 4 h. This was the same time required for *Helicoverpa zea* SNPV-*hsp70*/*lacZ* (HzSNPV-*hsp70*/*lacZ*) to express *lacZ* in fourth-instar *H. zea* and *H. virescens* larvae, and HzSNPV is considered to be the most virulent

baculovirus for heliothines (Washburn *et al.*, 2001). By comparison, the first detectable expression of *lacZ* by both AcMNPV AcCtlNt and Ac21/20 was 6 h in *T. ni* and 8 h in *H. virescens* (Washburn *et al.*, 2003a). The corresponding LD_{50} values of AcCtlNt in *S. exigua*, *T. ni* and *H. virescens* were 4, 6 and 6 occlusions, respectively, in insects inoculated at 4^0 and 8, 12 and 18 occlusions in insects inoculated as 4^{16} (Washburn *et al.*, 2003a). Thus, both the rapidity of early viral gene expression (as indicated by first expression of *lacZ* in the midgut) and the attenuated sloughing of infected midgut cells in *S. exigua* enhanced the oral virulence of AcMNPV. The earlier onset of *lacZ* expression in the midgut and the reduced rate of infected midgut cell sloughing are not independent factors; however, the rate of sloughing increases progressively with time within an instar. Infected midgut cell sloughing exerts strong selection pressure on the virus to transmit infection rapidly to secondary target cells, and early expression of *gp64* facilitates this process. Early expression of *gp64* had no effect on the timing of *lacZ* expression in midgut cells. The cost to AcMNPV in terms of increased inoculum required to establish an LD_{50} with virus that cannot express *gp64* early depended on relative rates of sloughing. Hence, in 4^{16} larvae, 4·4, 9·4, and 43·3 times more Ac21/20 was required to achieve an LD_{50} in *S. exigua*, *T. ni* and *H. virescens*, respectively, relative to AcCtlNt. Early expression of *gp64*, therefore, is an effective viral mechanism for overcoming developmental resistance, and the earlier the expression, the more effective it is.

Previously, Flipsen *et al.* (1995) constructed an AcMNPV double reporter recombinant in which *lacZ* was placed under the control of the functionally early *Drosophila melanogaster hsp70* promoter, and *β*-glucuronidase (*β-GUS*) was placed under the control of the very late AcMNPV *p10* promoter. They used this recombinant to study early pathogenesis in *S. exigua* larvae and found that *β*-galactosidase appeared in secondary target cells (undifferentiated midgut epithelial cells) before *β-GUS* was detected in ODV-infected midgut columnar cells. These results indicated that secondary targets were infected before virus replication and late gene expression had occurred in primary targets. Similarly, with wild-type GP64 synthesis, the lag time between the onset of *lacZ* expression within infected midgut and tracheal cells of *T. ni* and *S. exigua* was only 3–4 h. We previously reported similar results during the early stages of AcMNPV infection in the semi-permissive hosts *Manduca sexta* and *H. zea* (Washburn *et al.*, 1996, 2000). In cultured insect cells, it takes ~10–12 h for the *de novo* synthesis of AcMNPV BV. Thus, the results from these *in vivo* studies support the hypothesis that the earliest systemic infections by wild-type AcMNPV arise from ODV-derived nucleocapsids repackaged as BV via early GP64 synthesis. This repackaging phenomenon can only occur with NPVs having the M phenotype. The restriction of the MNPVs to species within the Lepidoptera suggests that evolutionary coupling of these two phenotypic traits has allowed these viruses to exploit larval lepidopterans by overcoming their first, and often only, line of defence, sloughing of infected midgut cells.

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