Early Synthesis of Budded Virus Envelope Fusion Protein GP64 Enhances *Autographa californica* Multicapsid Nucleopolyhedrovirus Virulence in Orally Infected *Heliothis virescens*

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Autographa californica **multicapsid nucleopolyhedrovirus (Ac*M*NPV), the type species of the *Nucleopolyhedrovirus* genus (*Baculoviridae* family), has two highly unusual traits shared by several baculovirus species. First, the occlusion-derived virus (ODV) that establishes primary infection in the midgut following its ingestion by host larvae contains multiple nucleocapsids, all of which enter the same midgut cell. Second, GP64, the envelope fusion protein of the budded virus (BV) that spreads infection beyond the midgut, is synthesized both early and late during infection. We tested the hypothesis that, together, these two traits enable parental ODV nucleocapsids to bud from infected midgut cells, essentially as BV, to establish secondary infections prior to completion of viral replication within the midgut. This "pass-through" strategy would enable the virus to counter the host's principal defense, sloughing of infected midgut cells, by accelerating the onset of systemic infections. To test this hypothesis, we created an Ac*M*NPV recombinant, AcLate21/20-64HB, that can express *gp64* only during the late phase of infection (coincident with the other structural proteins). We then compared the virulence of this virus to that of a control recombinant virus that expresses *gp64* in a wild-type manner. We found that when administered orally, the control virus was far more virulent and established secondary infection earlier than AcLate21/20-64HB, but when administered intrahemocoelically, infectivity and virulence of the two recombinants were identical. Our results demonstrate that early *gp64* expression is a key component of a unique and highly adaptive baculovirus infection strategy.**

Nucleopolyhedroviruses (NPVs) (family *Baculoviridae*) often produce fatal infections in their larval lepidopteran hosts. The NPVs that are not restricted to the midgut tissues of their hosts require two morphologically different forms to achieve this end. Infection is initially established in feeding larvae following ingestion of viral occlusions containing enveloped virions. These occlusions dissolve upon contact with the alkaline digestive juices within the midgut lumen of the larva, releasing the occlusion-derived virus (ODV) that subsequently initiates primary infection of mature columnar epithelial cells within the midgut. Budded virus (BV) produced in these cells initiates secondary infections that, in turn, are the source of additional BV that spreads infection throughout the host. Ultimately, nearly every tissue becomes infected, causing the host to succumb to baculovirus infection.

The NPVs have been divided into two groups on the basis of their phylogenetic relatedness (13). Group I NPVs share 17 genes not present in group II NPVs, one of which is *gp64*, the gene that encodes the BV envelope fusion protein (13). GP64 enables group I BVs to enter target cells productively; therefore, it is essential for infectivity both in vitro and in vivo (3, 23, 32). The BVs of group II NPVs use another surface glycoprotein, F (fusion), for entry (26, 38). Interestingly, group I NPVs also encode the F protein or its subdomains, suggesting, as Pearson et al. noted (25) that *gp64* was acquired by group I baculoviruses more recently than the F-encoding gene and that GP64 displaced the fusion function of the F protein (26). Following this logic, GP64 must be evolutionarily advantageous for the group I NPVs.

Autographa californica multicapsid NPV (Ac*M*NPV) is a group I NPV, the type species of the NPV genus and the best-studied baculovirus. Ac*M*NPV has a very broad host range that encompasses several lepidopteran families, suggesting that it has evolved an infection strategy highly effective at overcoming host defenses. One of the principal defenses of lepidopteran larvae against baculovirus infection is to slough ODV-infected midgut cells (5, 6, 17, 22, 34, 35, 37). Infected midgut cells are sloughed throughout larval development, and as larvae age within each instar they become increasingly resistant to fatal infection, a phenomenon known as developmental resistance (6, 8, 30, 35). Moreover, during each molt, infected midgut epithelial cells are shed into the gut lumen and voided as regenerative cells differentiate and form a larger tissue. Within each instar, therefore, the temporal window for establishing an Ac*M*NPV systemic infection becomes progressively smaller and ultimately closes during the molt to the next instar (6, 35). Hence, for Ac*M*NPV to kill permissive hosts, BV must bud from ODV-infected midgut cells and infect tracheal epidermal cells (the primary target of BV infection) within the

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insect's respiratory system before the infected midgut cells are sloughed.

The temporal production of GP64 differs from that of AcMNPV's other structural proteins in that GP64 synthesis begins during the early phase of infection, hours before viral DNA replication and morphogenesis of viral progeny (2, 20). Such early synthesis of a structural protein is unusual among viruses. Also unusual is the fact that ODV of AcMNPV and certain other group I and group II NPVs contain multiple nucleocapsids per virion (the M trait). Notably, the BV particles of these M viral species (which exclusively infect larval lepidopterans) contain only a single nucleocapsid. Because the cellular targets of AcMNPV ODV can be infected by virions containing a single nucleocapsid (36), packaging multiple nucleocapsids within a virion might appear to be wasteful (because all the nucleocapsids enter the same cell). However, this trait apparently has also been selected for over evolutionary time and therefore should be advantageous.

One possible advantage is that the extra nucleocapsids may provide extra copies of *trans*-acting genes to complement genes mutated by UV radiation (27). Another possibility, which does not exclude the first possibility, is that multiple-nucleocapsid ODV packaging and early expression of *gp64*, combined, contribute to improved viral fitness. In this scenario, a subpopulation of viral nucleocapsids is transported to the nucleus of an ODV-infected midgut cell, while a separate subpopulation is transcytosed directly to the basal plasma membrane. The nuclear subpopulation initiates *gp64* expression early during infection, and the protein is synthesized and transported to the basal plasma membrane, enabling the transcytosed population of nucleocapsids to bud. These repackaged ODV nucleocapsids could initiate a systemic infection hours before completion of de novo synthesis of viral progeny, thereby reducing the effectiveness of the host's defensive sloughing response. We tested the latter hypothesis empirically in larvae of the permissive host *Heliothis virescens* (Noctuidae) by comparing the infection efficiency and pathogenesis of AcCtlNt-64HB, an AcMNPV recombinant designed to produce GP64 in a temporally wild-type (WT) manner (early and late), with those of another recombinant, AcLate21/20-64HB, that synthesizes GP64 only late during infection, coincident with its other structural proteins. Compared to AcCtlNt-64HB, AcLate21/20-64HB established secondary infection much more slowly and was far less virulent when inoculated into larvae orally. In contrast, when BV was injected directly into the larval hemocoel, circumventing infection of the midgut, virulence of the two recombinants was identical. Our results demonstrate that early *gp64* expression, coupled with the M ODV phenotype, is a key component of a unique and highly adaptive baculovirus infection strategy.

MATERIALS AND METHODS

Construction and genetic analysis of *gp64* recombinant viruses. AcLate21/20-64HB was designed to express *gp64* solely during the late phase of infection. This recombinant retains only a fragment of the native early-late *gp64* promoter driving *gp64* expression [designated AcP(21/20) min by Garrity et al. (10)], which was reported to generate WT level of late, but not early, transcription of *gp64* (Fig. 1A and B). In addition to AcP(21/20) min, the *gp64* promoter of AcLate21/20-64HB includes 27 extraneous nucleotides that were introduced by the transfer plasmid, p64Late21/20-64+p24, used to construct this virus. To control for the potential influence of these extraneous nucleotides, we created a perfectly

matched recombinant control virus, AcCtlNt-64HB, by using another transfer plasmid, p64CtlNt-64+p24, that contains an intact early-late *gp64* promoter plus the same 27-nucleotide sequence found in p64Late21/20-64+p24 (Fig. 1C). Both transfer plasmids were constructed from p64KTV1 (19) through a series of intermediates in which most of *gp64* and *p24*, including all of the native *gp64* promoter, were deleted from the *Bgl*II site at position 108033 to the *Not*I site at position 110345 in the AcMNPV genome (1). The desired promoter sequences were then inserted as synthetic oligonucleotides, and the native *p24* and *gp64* coding sequences were restored by inserting sequence-verified, cloned PCR amplification products. The *Bgl*II and *Not*I sites marked the upstream and downstream junctions of these manipulations, respectively, and the *p24-gp64* junctions in both transfer plasmids were verified by direct sequence analysis (shown in Fig. 1).

In addition to AcLate21/20-64HB and AcCtlNt-64HB, a second control virus, designated AcCtl-64HB, that has a complete WT *gp64* promoter was engineered (Fig. 1A). This virus was constructed to ascertain if the extraneous 27 nucleotides in AcCtlNt-64HB affected infection in vitro or in vivo. All of the recombinant viruses were isolated by cotransfecting Sf9 cells with p64Late21/20-64+p24, p64CtlNt-64+p24, or p64KTV1 and *Bsu*36I-digested genomic DNA from another AcMNPV (strain E2) recombinant, AcDCHspBlue. This virus has two *Bsu*36I sites within *gp64* and an *Escherichia coli lacZ* reporter gene (driven by the *Drosophila melanogaster hsp70* promoter) inserted at the *Eco*RV site upstream of the intact *polyhedrin* gene (1). We engineered the *hsp70/lacZ* cassette into our recombinants so we could monitor the course of infection in vivo (7). Most of the progeny viruses produced by our cotransfected cells were expected to have *gp64* genes derived from the transfer plasmids because *Bsu*36I digestion deletes most of this essential gene from the parental viral DNA. Progeny viruses were harvested from each cotransfection, resolved by plaque assays on Sf9 cells, amplified, and then screened by PCR analysis of viral DNA minipreps (24).

The recombinant viral clones chosen for the studies described here were subjected to two additional cycles of plaque purification and amplified, and the titers of the recombinant viruses were determined by plaque assays using Sf9 cells. Subsequently, the genetic structure of the *p24-gp64* region in each recombinant was verified using standard PCR (16) and Southern blotting (28) analyses. The viral DNAs used for these analyses were extracted from partially purified BV samples as described previously (24). The PCRs were performed with various gene-specific primer pairs under standard conditions (described in reference 16), and the products were harvested and analyzed by 1% agarose gel electrophoresis with ethidium bromide staining. The sequences of the primers used for these analyses were as follows: primer 1, 5′-GCTCAACATCATAAACGTGTCCG-3′; primer 2, 5′-TGCGCGTTGCAGTGCTCC-3′; primer 3, 5′-GATAGCCATCG TGATCGC-3′; and primer 4, (5′-GAACTTGCGTCTACTGCAGTC-3′).

For Southern blotting analyses, viral genomic DNAs were digested with various restriction endonucleases, and the resulting genomic fragments were resolved on agarose gels, transferred to positively charged membranes (Zetaprobe; Bio-Rad Laboratories, Hercules, Calif.) under alkaline conditions, and hybridized with gel-purified, uniformly labeled DNA probes, as described previously (18).

Nucleocapsid packaging within ODV of AcCtlNt-64HB and AcLate21/20-64HB. To determine if the virion packaging characteristics of AcCtlNt-64HB and AcLate21/20-64HB were similar, we used transmission electron microscopy (TEM) to quantify the numbers of nucleocapsids per ODV and ODVs per occlusion for both recombinants. Occlusions were prepared for TEM by standard protocols, and for each virus, we examined sections of 100 individual occlusions at a magnification of ×10,500, scoring only the ODVs in which the nucleocapsids were sectioned perpendicular to the viewing plane. To assess the numbers of ODV per occlusion, we printed photographs of sections (magnification of ×15,000) on standardized paper and cut out the images of individual occlusions. The paper image of each occlusion cross section was then weighed, and the number of visible ODV was recorded. Our rationale for using weight as a measure of area was based on the fact that the paper images are essentially two dimensional, as is area. Indeed, weight provided a highly accurate estimate for occlusion cross-sectional area ($r^2 = 0.94$; data not shown) and, therefore, allowed us to correlate weight (as a measure of occlusion cross-sectional area) with the numbers of ODV.

In vitro analysis of recombinant baculoviruses. Several different assays were performed to compare the behavior of AcLate21/20-64HB with strain E2 (WT AcMNPV), AcCtl-64HB, and AcCtlNt-64HB during in vitro infection of Sf9 cells. Each virus was used to infect individual batches of Sf9 cells at a multiplicity of infection of 5 to 10 PFU per cell, the inocula were removed, and the infected cells were washed and resuspended in fresh medium. The infected-cell cultures were then aliquoted into six-well plates (Corning Glass Works, Corning, N.Y.) at a density of 10^6 cells/well and incubated for various times at 28°C.

A. AcMNPV WT and AcCtl-64HB:

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                                    ←[gp64 ATG]           ←[gp64 L4]
109701  ACAATAGCGC TTACCATCTT GCTTGTGTGT TCCTTATTGA AGCCTTGGTG

            ←[gp64 E]                        ←[gp64 L3]
109751  TGACTGATTT ACTAGTAGCA TTGAGGCATC TTATATACCC GACCGTTATC

                                                      ←[gp64 L2]
109801  TGGCCTACGT GACACAAGGC ACGTTGTTAG ATTAATAATC TTATCTTTTT

            ←[gp64 L1]
109851  ATCTTAATTG ATAAGATTAT TTTTATCTGG CTGTTATAAA AACGGGATCA
            [p24 L]→                                   [p24 ATG]→

109901  TGAACACGGA CGCTCAGTCG ACATCGAACA CGCGCAACTT CATGTACTCT
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B. AcLate21/20-64HB:

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                                    ←[gp64 ATG]           ←[gp64 L2]
109701  ACAATAGCGC TTACCATCTT agatctCACG TTGTTAGATT AATAATCTTA

                    ←[gp64 L1]      Not I          ←[gp64 L1 DUP]
109751  TCTTTTTATC TTAATTGATA AGcggccgct ttttatctta attgataagA
                    [p24 L]→                       [p24 L DUP]→

109801  TTATTTTTAT CTGGCTGTTA TAAAAACGGG ATCATGAACA CGGACGCTCA
                                            [p24 ATG]→

109851  GTCGACATCG AACACGCGCA ACTTCATGTA CTCT
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C. AcCtlNt-64HB:

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                                    ←[gp64 ATG]           ←[gp64 L4]
109701  ACAATAGCGC TTACCATCTT GCTTGTGTGT TCCTTATTGA AGCCTTGGTG

            ←[gp64 E]                        ←[gp64 L3]
109751  TGACTGATTT ACTAGTAGCA TTGAGGCATC TTATATACCC GACCGTTATC

                                                      ←[gp64 L2]
109801  TGGCCTACGT GACACAAGGC ACGTTGTTAG ATTAATAATC TTATCTTTTT

            ←[gp64 L1]      NotI            ←[gp64 L1 DUP]
109851  ATCTTAATTG ATAAGcggcc gcttttttatc ttaattgata agATTATTTT
            [p24 L]→                       [p24 L DUP]→

109901  TATCTGGCTG TTATAAAAAC GGGATCATGA ACACGGACGC TCAGTCGACA
                                    [p24 ATG]→

109951  TCGAACACGC GCAACTTCAT GTACTCT
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FIG. 1. Promoter regions of the *gp64* genes in WT Ac*M*NPV and recombinant viruses constructed for this study. (A) Early-late *gp64* promoter in WT Ac*M*NPVand AcCtl-64HB; (B) minimal late *gp64* promoter plus extraneous sequence in AcLate21/20-64HB; (C) early-late *gp64* promoter plus extraneous sequence in AcCtlNt-64HB. Early (E) and late (L) transcriptional motifs and the translational initiation site in the *gp64* gene are singly underlined; the late (L) transcriptional motif and translational initiation site in the adjacent *p24* gene are doubly underlined. The 21/20 minimal late *gp64* promoter sequence around L2 (10) is italicized. An extraneous sequence introduced during the construction of modified *gp64* promoter regions is underlined with a wavy line (B and C). All sequences are presented in the orientation of the Ac*M*NPV genome, and the numbers on the left are derived from the viral genome sequence (1). DUP, duplication.

Radioimmunoprecipitation and immunoblotting assays were performed to examine biosynthesis and accumulation of GP64, respectively. For the radioimmunoprecipitation experiments, the infected cells were pulse-labeled for 4 h at various times after infection, intracellular and extracellular fractions were pre-pared and combined, and then samples from each time point were immunoprecipitated using GP64-specific (AcV1 [14]) or control (PAb405 [12]) monoclonal antibodies, as described previously (20).

For the immunoblotting experiments, intracellular and extracellular fractions

were prepared and combined at various times after infection. Then samples from each time point were resolved by discontinuous sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE), transferred to Immobilon membranes (Millipore Corporation, Bedford, Mass.), and probed with a polyclonal, GP64-specific rabbit antiserum, as described previously (21).

One-step growth curve assays were performed to examine viral replication in vitro. For these assays, the media from triplicate wells were harvested at various times postinfection, mixed, and clarified by low-speed centrifugation in a clinical centrifuge, and infectious BV was measured by plaque assays on Sf9 cells, as described previously (29).

In vivo studies. For all in vivo experiments, we used fourth-instar larvae of *H. virescens* reared on a modified wheat germ diet (Stoneville). Each larva was individually inoculated with occlusions or BV of WT AcMNPV (strain E2), AcCtl-64HB, AcCtlNt-64HB, or AcLate21/20-64HB in 1-μl aliquots using a microapplicator (Burkhard) fitted with a blunt- or sharp-tip 32-gauge needle, respectively, mounted on a 1-ml tuberculin syringe (7). Occlusions were administered orally, and BV was injected into the hemocoel by inserting the needle through the planta of one of the prolegs (7, 34). Larvae were orally inoculated within 15 min after shedding the third-instar cuticle (i.e., newly molted larvae) or 16 h after the molt. For all intrahemocoelic inoculations, larvae were maintained at 28°C after molting to the fourth instar and inoculated 24 h (±6 h) later. Following inoculation, all larvae were maintained in a growth chamber at 28°C ± 2°C under constant illumination in individual 25-ml cups with Stoneville diet provided ad libitum.

For bioassays, larvae were maintained in individual cups until death or pupation; death from polyhedrosis diseases was confirmed by the presence of occlusions in the cadavers. In time course experiments, large numbers of newly molted larvae were orally inoculated with either AcCtlNt-64HB or AcLate21/20-64HB and maintained as described above. Throughout the fourth instar, we sacrificed larval cohorts at 4-h intervals, removed the midguts and associated tracheal elements from the larvae, and then processed and examined these tissues microscopically for the blue *lacZ* signals which serve to identify infected cells (7, 34).

RESULTS

Genetic analysis of recombinant baculoviruses. Three recombinant viruses, all containing *lacZ* reporter genes driven by the *Drosophila hsp70* promoter, were produced for this study. They are AcLate21/20-64HB, which can express *gp64* only during the late phase of infection, and AcCtlNt-64HB and AcCtl-64HB, which are control viruses that like WT AcMNPV, express *gp64* during the early and late phases of infection. The *gp64* promoter in AcCtl-64HB is WT, but in AcCtlNt-64HB, it contains an additional 27-nucleotide sequence to control for the presence of this sequence in the *gp64* promoter of AcLate21/20-64HB (Fig. 1).

PCRs, using a primer pair designed to amplify a 1,068-bp fragment that includes the native *gp64* promoter, produced slightly smaller and slightly larger amplimers when AcLate21/20-64HB and AcCtlNt-64HB viral DNAs were used as the templates, respectively (Fig. 2, lanes 1 to 5). Similar results were obtained using a different primer pair designed to amplify the entire *gp64* gene (including the promoter and open reading frame) in each virus, though the size differences were not as evident due to the larger size of the amplimer (Fig. 2, lanes 6 to 10). Note that the latter amplimers were digested with *Bsu*36I prior to electrophoresis to confirm the loss of *Bsu*36I sites from the parental virus in these recombinants (Fig. 2, lanes 6 to 10).

As with the PCR analyses, the expected size differences in the *gp64* genes of WT AcMNPV, AcCtl-64HB, AcCtlNt-64HB, and AcLate21/20-64HB were also observed when Southern blots of *Bam*HI-digested viral DNAs were probed with the 1,431-bp *Sal*I fragment of WT AcMNPV (Fig. 3A). In addition, these results showed that the *p24-gp64* genes in the transfer
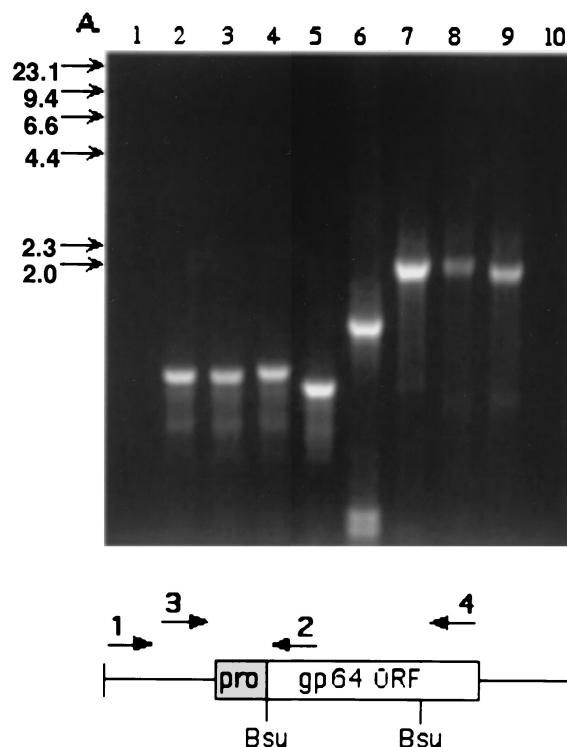

FIG. 2. PCR and agarose gel electrophoresis analysis of recombinant viral DNAs. Primers 1 and 2 were designed to amplify the *gp64* promoter region from each virus (lanes 1 to 5), whereas primers 3 and 4 were designed to amplify the entire *gp64* gene, including the promoter and open reading frame, from each virus (lanes 6 to 10). The products obtained with primers 3 and 4 were digested with *Bsu*36I prior to electrophoresis to distinguish between the parental (Ac64DCHspBlue) and recombinant viruses. The drawing below the gel shows the approximate locations of the primer sequences (numbered arrows), *pro* gene, *gp64* open reading frame (ORF), and *Bsu*36I (*Bsu*) sites in the parental virus. Lanes 1 and 10, no DNA; lane 2, AcMNPV; lanes 3 and 7, AcCtl-64HB; lanes 4 and 8, AcCtlNt-64HB; lanes 5 and 9, AcLate21/20-64HB, lane 6, Ac64DCHspBlue. The positions of molecular size markers (in kilobases) are indicated to the left of the blot.

plasmids had recombined at the correct genomic location. The recombinants were also clearly distinguished when Southern blots of *Not*I-digested viral DNAs were probed with a WT *p24-gp64* fragment (Fig. 3B). This fragment hybridized to a single 1,467-bp fragment from WT AcMNPV and AcCtl-64HB, to 500- and 1,040-bp fragments from AcCtlNt-64HB, and to 500- and 950-bp fragments from AcLate21/20-64HB. These results were expected because each of the last two recombinants should have had the additional *Not*I site found in the *p24-gp64* promoter junction of their respective transfer plasmids (Fig. 1). Finally, only the expected differences were found when we analyzed *Sal*I- or *Hin*dIII-digested WT AcMNPV, AcCtl-64HB, AcCtlNt-64HB, and AcLate21/20-64HB viral DNAs by agarose gel electrophoresis and ethidium bromide staining (data not shown). Thus, the recombinant viruses isolated for this study had the expected genetic structures in the *p24-gp64* region and no other detectable alterations. Similar experiments verified the genetic structures of the *polyhedrin-*
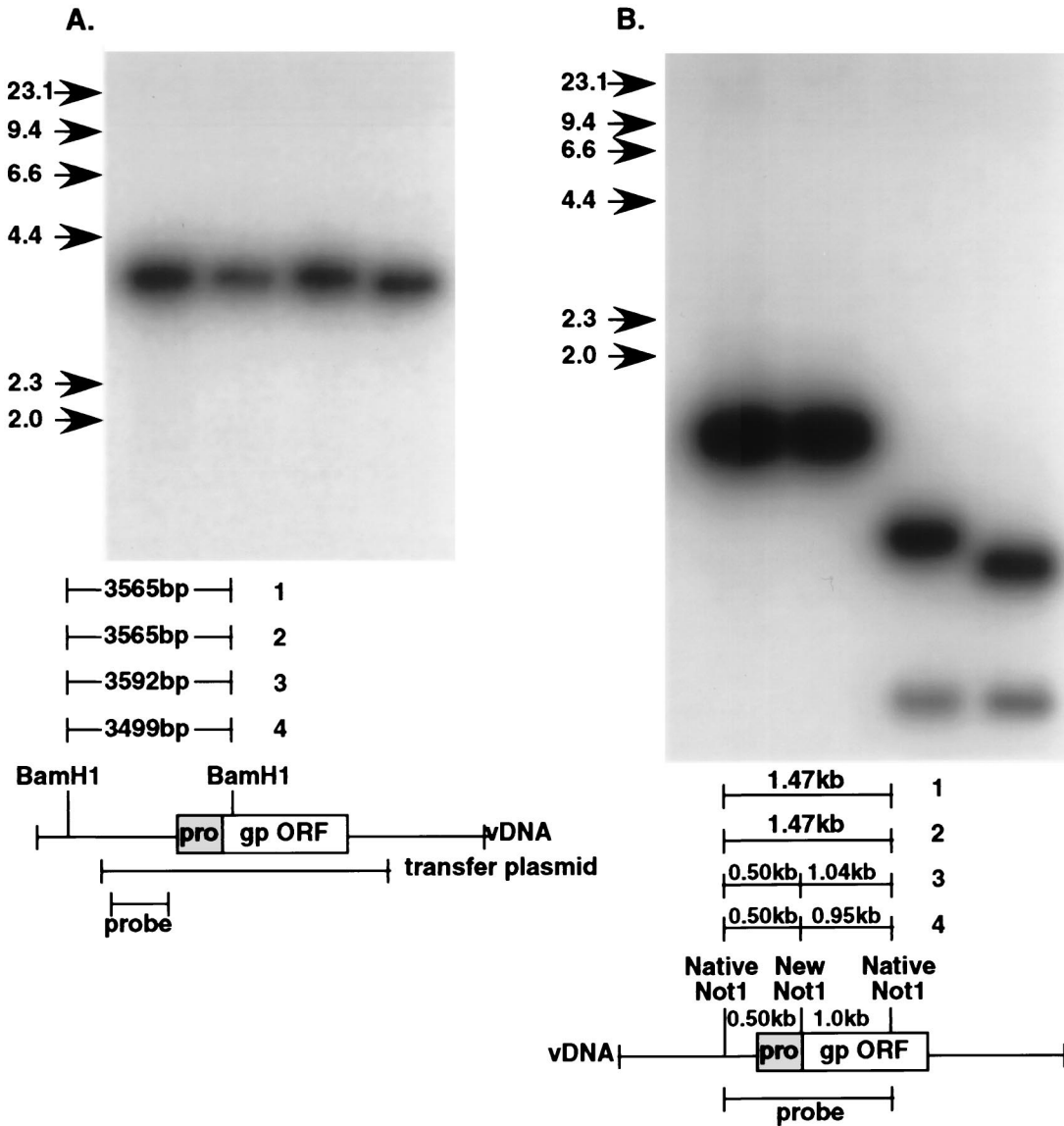
FIG. 3. Southern blotting analysis of recombinant viral DNAs. WT Ac*M*NPV (lanes 1) and recombinant viruses AcCtl-64HB (lanes 2), AcCtlNt-64HB (lanes 3), and AcLate21/20-64HB (lanes 4) were studied. Genomic DNAs isolated from the various recombinant baculoviruses produced for this study were digested with *Bam*HI (A) or *Not*I (B), and the digests were analyzed by Southern blotting (28) as described in Materials and Methods. The positions of molecular size markers (in kilobases) are indicated to the left of the blots. The probes used for the blots shown in panels A and B were the 1,431-bp *Sal*I fragment or the 1,467-bp *Not*I fragment derived from the *gp64* region of the Ac*M*NPV genome, as shown in the drawings beneath the blots. The locations of the primers (primers 1 to 4), *Bam*HI and *Not*I restriction sites, the *pro* gene, the *gp64* open reading frame (ORF), and the probe on the viral DNA (vDNA) are shown.

hsp-lacZ region in AcDCHspBlue and of the recombinants derived from this parent (data not shown).

GP64 synthesis and accumulation and in vitro growth characteristics of WT Ac*M*NPV, AcCtl-64HB, AcCtlNt-64HB, and AcLate21/20-64HB. To assess GP64 synthesis patterns and BV production, we compared the biological properties of WT Ac*M*NPV, AcCtl-64HB, AcCtlNt-64HB, and AcLate21/20-64HB in cultured Sf9 cells. The results of radioimmunoprecipitation experiments showed that GP64 was produced as early as 1 to 4 h postinoculation (hpi) in cells infected with either WT Ac*M*NPV, AcCtl-64HB, or AcCtlNt-64HB as expected (20) (Fig. 4A). In contrast, GP64 production by AcLate21/20-64HB

was not detected until 8 to 12 hpi (Fig. 4A). Similarly, immunoblotting assays revealed that GP64 was first detected at 8 hpi in WT Ac*M*NPV-, AcCtl-64HB-, and AcCtlNt-64HB-infected Sf9 cells, but not until 18 hpi in cells infected by AcLate21/20-64HB (Fig. 4B). By 24 hpi, the biosynthesis rates and accumulated amounts of GP64 were approximately the same for all four viruses. These results demonstrated that the *gp64* early promoter element had been eliminated from AcLate21/20-64HB without compromising late promoter function. Additionally, the absence of any detectable differences in *gp64* expression by WT Ac*M*NPV, AcCtl-64HB, and AcCtlNt-64HB showed that the extraneous 27-nucleotide sequence engi-
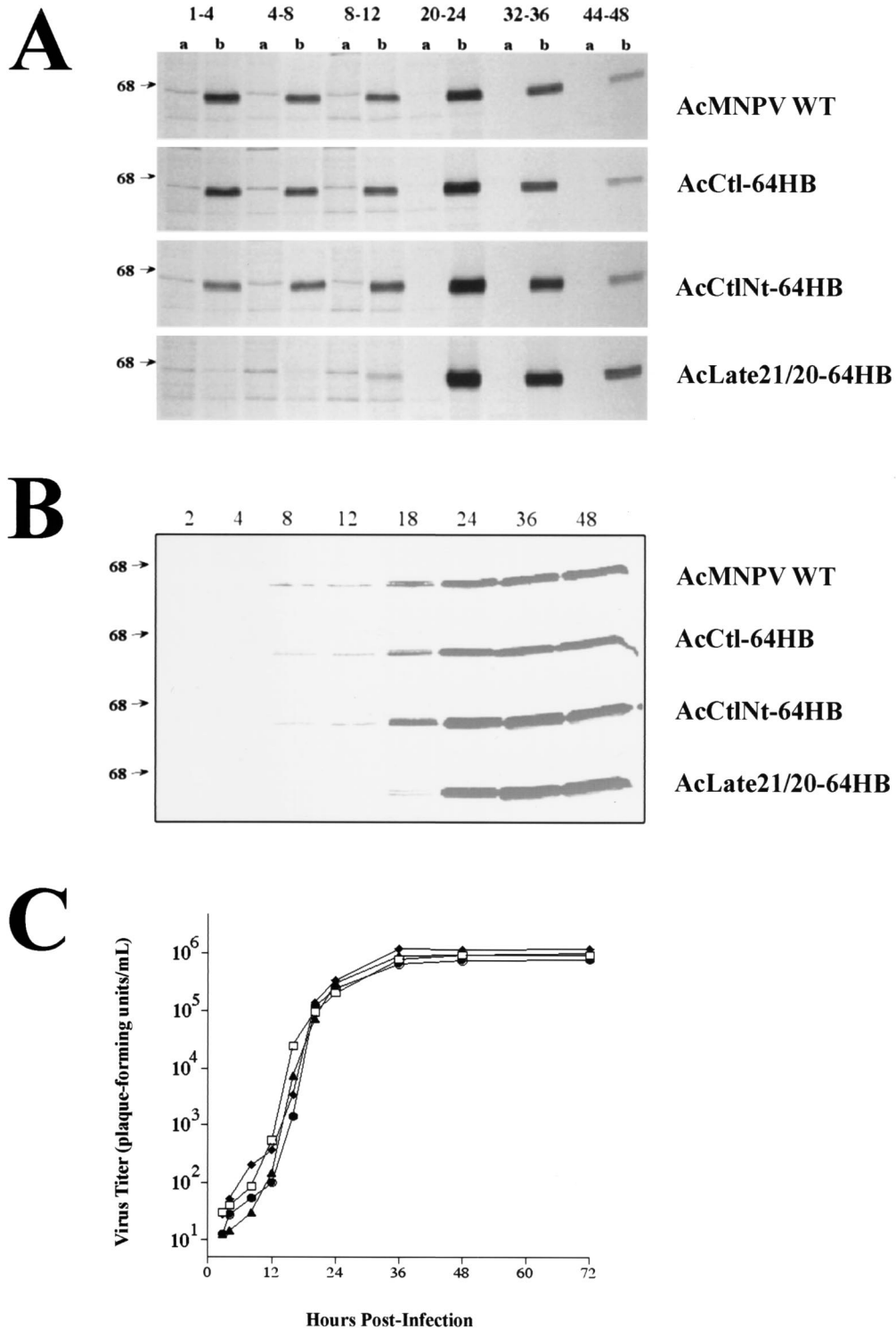
FIG. 4. In vitro analyses of the viruses used in this study. (A) Radioimmunoprecipitation of GP64. Sf9 cells were infected with the viruses shown to the right of the blots and radiolabeled for 4-h intervals, and intracellular and extracellular fractions were prepared and combined at the indicated times (in hours) postinfection. The samples were then split and immunoprecipitated with either a control monoclonal antibody (lanes a) or monoclonal antibody against GP64 (lanes b). Washed immunoprecipitates were disrupted and analyzed by SDS-PAGE and autoradiography. The position of the 68-kb molecular size marker is shown to the left of the blots. (B) Immunoblots comparing GP64 accumulation. Sf9 cells were infected with the indicated viruses, intracellular and extracellular fractions were prepared and combined at the indicated times (in hours) postinfection, and samples were analyzed by SDS-PAGE and immunoblotted with antibody to GP64. (C) One-step growth curves of WT AcMNPV (white boxes), AcCtl-64HB (black diamonds), AcLate21/20-64HB (black triangles), and AcCtlNt-64HB (black circles). Cell-free media were harvested at various times postinfection, replicate medium samples were mixed, and the titers of progeny virus were determined by duplicate plaque assays on Sf9 cells. The plot shows the average viral titers.

neered into the *gp64* promoter of AcLate21/20-64HB (and AcCtlNT-64HB) had no discernible impact on *gp64* expression. Finally, one-step growth curves of the WT and recombinant viruses revealed no differences in either the timing or level of infectious BV progeny production, demonstrating that the early wave of *gp64* expression is dispensable for normal in vitro replication of Ac*M*NPV in cultured Sf9 cells (Fig. 4C).

ODV packaging of AcLate21/20-64HB and AcCtlNt-64HB. We determined the number of nucleocapsids in sections taken from 256 and 262 ODVs of AcCtlNt-64HB and AcLate21/20-64HB, respectively, by TEM. The mean number of nucleocapsids per ODV was the same for each virus (5.7 ± 2.1 for AcCtlNt-64HB and 5.4 ± 2.1 for AcLate21/20-64HB), as was the distribution of the number of nucleocapsids per ODV (Fig. 5A). Similarly, the correlation between the number of ODV and the relative cross sectional area of occlusions was linear and virtually identical for AcCtlNt-64HB and AcLate 21/20-64HB (Fig. 5B). In addition, the size ranges of occlusion cross sections were identical for both recombinants. Thus, we detected no differences between the ODV packaging of AcCtlNt-64HB and AcLate21/20-64HB.

Bioassays. In oral bioassays using two developmental cohorts of fourth-instar *H. virescens*, WT Ac*M*NPV, AcCtl-64HB, and AcCtlNt-64HB exhibited identical dose-mortality relationships (data not shown), demonstrating that the extra 27 nucleotides did not affect oral infectivity. By contrast, AcLate21/20-64HB was significantly less efficient at establishing fatal infections. The estimated 50% lethal doses for AcCtlNt-64HB orally inoculated into newly molted and fourth-instar larvae 16 h postmolt were 6 and 18 occlusions (Fig. 6A and C), respectively, compared to 16 and 780 occlusions, respectively, for AcLate21/20-64HB (Fig. 6B and D). Hence, in both developmental cohorts, eliminating early GP64 synthesis reduced the ability of Ac*M*NPV to infect host larvae fatally. Additionally, while both viruses established fatal infections less efficiently when inoculated 16 h later in the instar (due to developmental resistance), AcLate21/20-64HB was far less efficient than AcCtlNt-64HB. Compared to newly molted insects, larvae inoculated 16 h after molting required threefold-more AcCtlNt-64HB occlusions to achieve 50% mortality (Fig. 6A and C), whereas nearly 49-fold-more occlusions of AcLate21/20-64HB were required for 50% mortality (Fig. 6B and D). These findings demonstrate that a delay in GP64 synthesis reduces Ac*M*NPV virulence in vivo, most dramatically in hosts that are closer to molting.

To confirm that these results reflect midgut-related phenomena, we conducted additional bioassays in which BV of the two recombinants (or WT Ac*M*NPV) was inoculated directly into the hemocoels of mid-fourth-instar larvae, bypassing primary infection of the midgut. In contrast to the results obtained with oral inoculations, WT Ac*M*NPV, AcCtlNt-64HB, and AcLate21/20-64HB produced similar mortality levels. In all cases, the larvae were exquisitely sensitive, with only 0.02 PFU (~2.6 physical particles [33]) yielding 28 to 38% mortality. These results showed clearly that the loss of early *gp64* synthesis was inconsequential to BV production in vitro and to virulence in vivo when the natural route of infection via the midgut was bypassed.

Pathogenesis of AcCtlNt-64HB and AcLate21/20-64HB. To evaluate the effects of temporal differences in GP64 synthesis
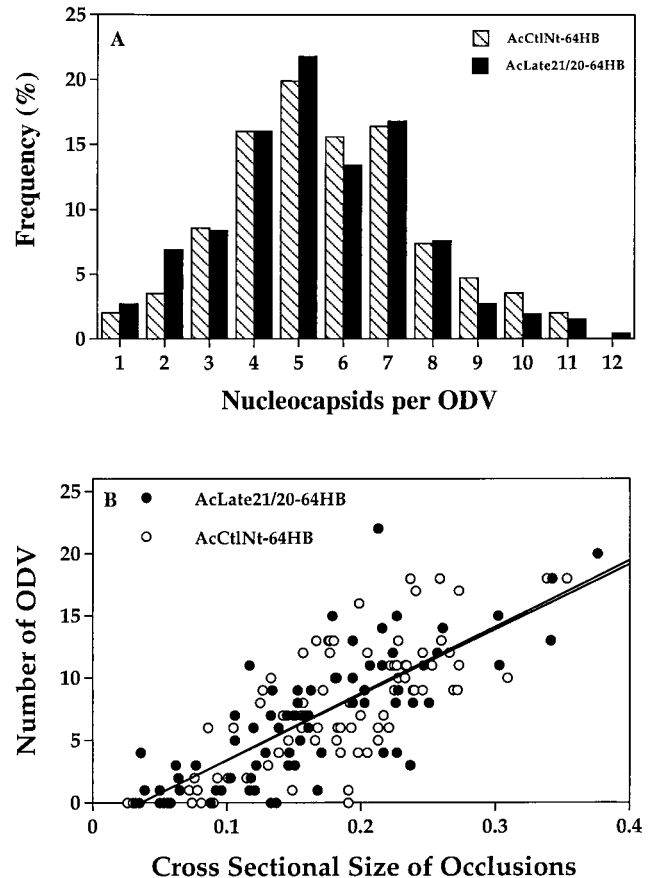

FIG. 5. (A) Frequency distribution of the number of nucleocapsids per ODV for 256 virions of AcCtlNt-64HB and 262 virions of AcLate21/20-64HB. (B) Correlation between the number of ODV particles and the cross sectional size of AcCtlNt-64HB and AcLate21/20-64HB occlusions. Occlusion size was determined as described in Materials and Methods. Regression lines were fitted by the least-squares method and are as follows: for AcCtlNt-64HB, $y = 52.6x - 1.9$ ($r^2 = 0.60$) ($n = 80$); for AcLate21/20-64HB, $y = 53.6x - 1.9$ ($r^2 = 0.54$) ($n = 73$).

on pathogenesis in vivo, the *lacZ* gene product was used to monitor the progression of AcCtlNt-64HB and AcLate21/20-64HB infections in *H. virescens* larvae. In this experiment, newly molted larvae were orally inoculated with 12 occlusions of AcCtlNt-64HB or 26 occlusions of AcLate21/20-64HB, dosages that yielded the same final mortalities (mean mortality for AcCtlNt of 88% ± 4% [standard error] [$n = 7$]; mean mortality for AcLate21/20 of 89% ± 2% [$n = 6$]). For both AcCtlNt-64HB and AcLate21/20-64HB, *lacZ* expression in midgut columnar cells was first observed at 8 hpi, indicating that the ODV of both viruses initiated gene expression in primary cellular targets at the same time (Fig. 7A). Subsequently, the percentage of *lacZ*-positive larvae increased for both viruses until 32 hpi, at which time the value was predictive of the final mortality levels. The number of infected-cell foci observed with both recombinants also increased during the first 20 to 24 hpi (Fig. 7B), reflecting an extended period during which new primary foci of infection were established. As expected, on the basis of identical ODV packaging characteristics (Fig. 5), inoculation with the larger number of AcLate21/20-
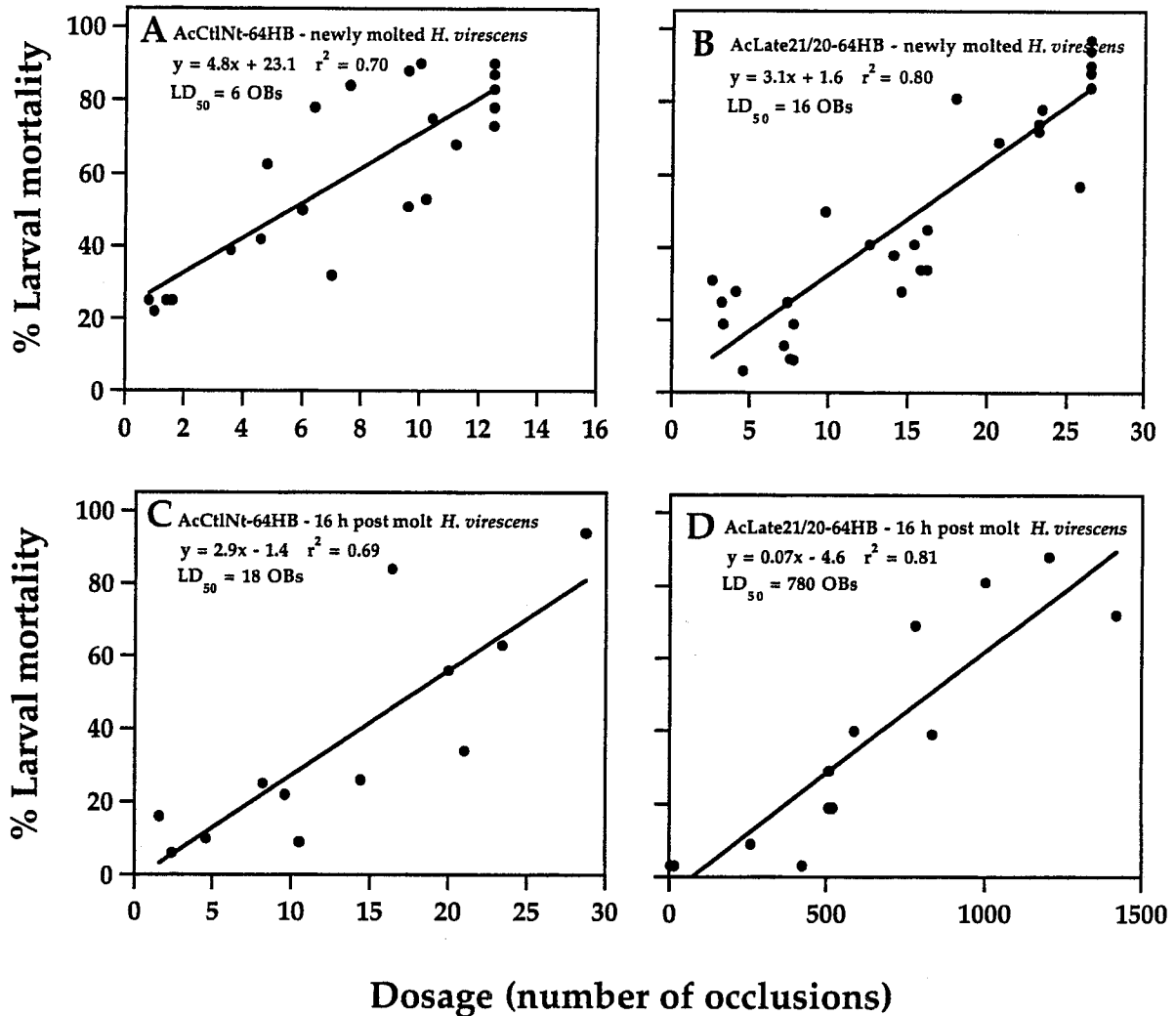
A AcCtlNt-64HB - newly molted *H. virescens*
$y = 4.8x + 23.1$ $r^2 = 0.70$
$LD_{50} = 6$ OBs

B AcLate21/20-64HB - newly molted *H. virescens*
$y = 3.1x + 1.6$ $r^2 = 0.80$
$LD_{50} = 16$ OBs

C AcCtlNt-64HB - 16 h post molt *H. virescens*
$y = 2.9x - 1.4$ $r^2 = 0.69$
$LD_{50} = 18$ OBs

D AcLate21/20-64HB - 16 h post molt *H. virescens*
$y = 0.07x - 4.6$ $r^2 = 0.81$
$LD_{50} = 780$ OBs

Dosage (number of occlusions)

FIG. 6. Dose-mortality relationships for *H. virescens* larvae inoculated orally as newly molted fourth instars (A and B) or 16 h after the molt (C and D) with occlusions of either AcCtlNt-64HB or AcLate21/20-64HB. Each point represents the percentage of a cohort containing between 28 and 32 insects that died from infection while in the larval stage. Regression lines were determined by the least-squares method and were used to calculate 50% lethal doses (LD_{50}s) (occlusions [OBs]).

64HB occlusions produced more foci (Fig. 7B). Even so, *lacZ* signals in tracheal cells, indicative of viral spread into secondary target tissues and establishment of systemic infection within the host's hemocoel, appeared earlier in AcCtlNt-64HB-infected insects than in AcLate21/20-64HB-infected insects (Fig. 7C). Statistical analysis by simple linear regression showed that the BV transmission rates of both viruses from midgut to tracheal cells were equivalent, but the onset of tracheal infection by AcCtlNt-64HB occurred 4.6 h earlier (Fig. 7C). This result is consistent with the interpretation that the earliest secondary infections by AcCtlNt-64HB were initiated by ODV nucleocapsids that had been repackaged as BV (via early GP64 synthesis), whereas those of AcLate21/20-64HB were initiated by BV synthesized de novo in primary cellular targets (following late expression of *gp64*).

Because AcLate21/20-64HB BV transmission from primary foci was delayed, the percentage of *H. virescens* larvae system-ically infected by this recombinant virus also lagged behind that of AcCtlNt-64HB at 12 hpi, but in cohorts sampled at 16 hpi and later, the percentages were similar (Fig. 7D). At 32 hpi, coincident with the onset of molting, all *lacZ*-positive larvae from both viral treatments had at least one focus of infection within the tracheal epidermis. Consistent with this observation, the proportions of *lacZ*-positive larvae in this sample were predictive of the final mortalities.

The composition of infected-cell foci also reflected the slower movement of AcLate21/20-64HB out of the midgut and indicated that significant numbers of midgut cells infected by ODV of either virus had been sloughed prior to the onset of the molt to the fifth instar (Fig. 8). For both recombinants, increasingly greater proportions of foci in older larvae consisted of infected tracheal epidermal cells only, but the frequency of these foci was lower in larvae infected with AcLate21/20. Because tracheal infections can be initiated only
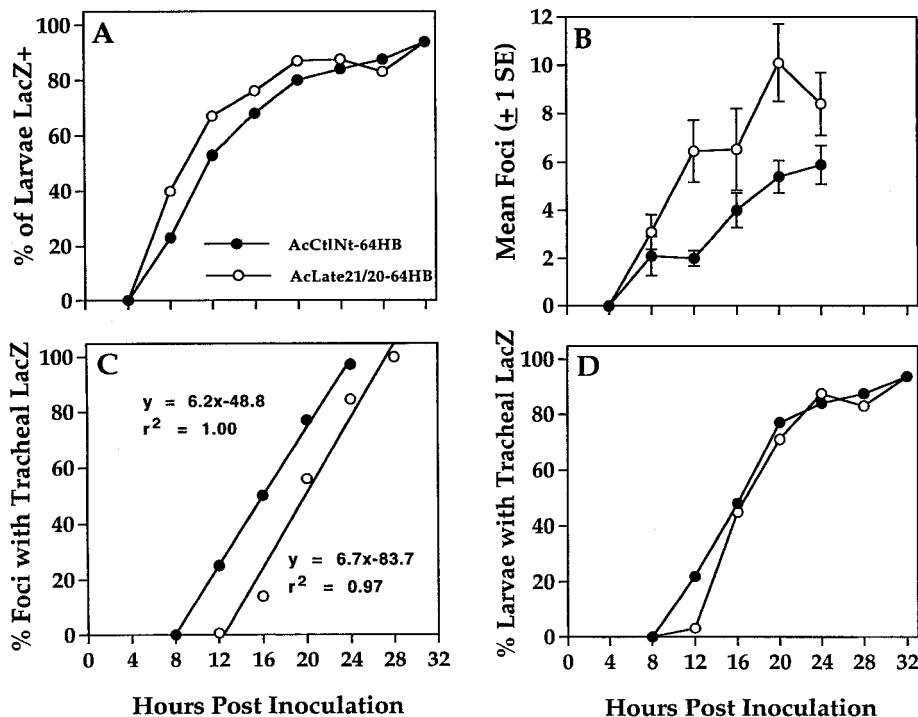
FIG. 7. Ac*M*NPV pathogenesis in *H. virescens* following oral inoculation of newly molted fourth-instar larvae with either 12 occlusions of AcCtlNt-64HB or 26 occlusions of AcLate21/20-64HB. Each point represents data from a cohort of between 29 and 32 larvae. (A) Percentage of larvae with *lacZ* expression in tissues at various times (in hours) postinoculation. (B) Mean number of foci per *lacZ*-positive larva; each error bar represents 1 standard error of the mean (SE). Foci numbers are not reported for 28 and 32 hpi, because at these time points, some viral plaques were contiguous, preventing accurate determination of foci numbers. (C) Percentage of viral foci containing one or more *lacZ*-positive tracheal epidermal cells; lines were determined by the least-squares method. (D) Percentage of larvae containing one or more *lacZ*-positive tracheal epidermal cells.

by BV produced by ODV-infected midgut cells, the primary cellular targets initiating these foci must have been sloughed previously (6, 22, 35). The somewhat lower frequency of such foci in larvae inoculated with AcLate21/20-64HB (Fig. 8) likely resulted from delayed movement of BV out of the midgut into immediate secondary cellular targets (tracheal cells) due to the delay in GP64 synthesis. If this is the case, then it follows that the midgut cells infected by AcLate21/20-64HB were more likely to be sloughed prior to establishing secondary infection than those infected by AcCtlNt-64HB. This result was consistent with our finding that higher dosages and increased numbers of primary foci of AcLate21/20-64HB were required to achieve the same levels of systemic infection and mortality as those of AcCtlNt-64HB.

DISCUSSION

Results from this study demonstrate that early synthesis of GP64 greatly enhanced the virulence of Ac*M*NPV in orally infected larvae of the permissive host *H. virescens*. By contrast, early GP64 synthesis was inconsequential for Ac*M*NPV infection of both cultured insect cells and larvae infected intrahemocoelically. Thus, an advantage for early synthesis of GP64 was observed only when larvae were infected by the natural route, orally, where the midgut was the primary target tissue. Data from the time course experiments showed that early GP64 synthesis specifically accelerated the transmission of BV

to tracheolar cells and by doing so, enhanced virulence by countering one of the host's principal means of defense, sloughing ODV-infected midgut cells (6, 15, 22, 34, 35). Sloughing of midgut cells is a well-documented physiological mechanism whereby caterpillars protect themselves during development. The vast majority of larval lepidopterans are herbivorous, and their food plants often contain both physical and chemical defenses that can reduce larval feeding efficiency. To satisfy nutritional requirements, caterpillars typically consume large quantities of plant materials, including cultivated crops; hence, they can be significant agricultural pests. During their extensive feeding bouts, caterpillars have a high probability of ingesting microbial pathogens, including baculoviruses, and shedding midgut epithelial cells is one way they can clear infections. Sloughing, therefore, may also be a strong selective force shaping the life history of Ac*M*NPV and other baculoviral species that must traverse the midgut in order to establish systemic infection. The payoff for successfully countering this host response is high, as shown by the extreme susceptibility of permissive species to BV entry into the larval hemocoel.

Over 20 years ago, Granados and Lawler (11) reported finding BV in the hemolymph of *Trichoplusia ni* less than an hour after oral inoculation with occlusions of Ac*M*NPV. This was the first documentation of astonishingly rapid production of BV by infected midgut cells, and the subsequent elucidation of the temporal regulation of GP64 transcription provided a plau-
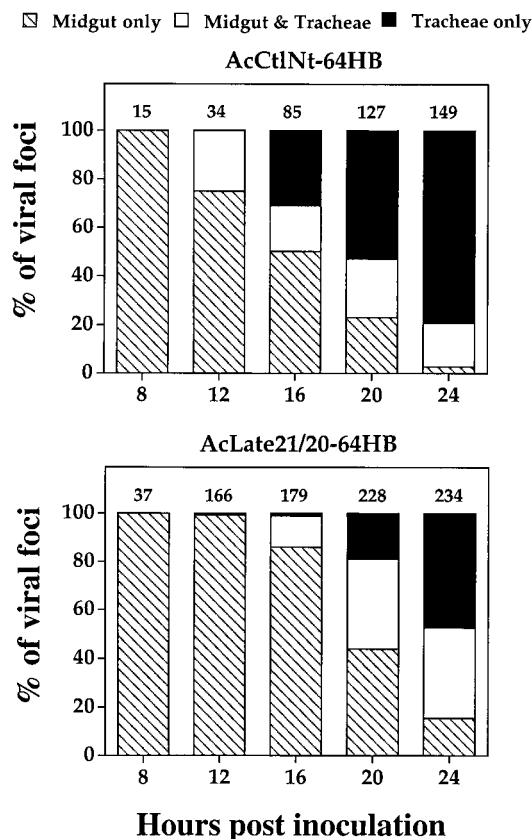
Midgut only ☐ Midgut & Tracheae ■ Tracheae only

FIG. 8. Cellular composition of viral foci in *H. virescens* orally challenged with 12 occlusions of AcCtlNt-64HB or 26 occlusions of AcLate21/20-64HB. Each bar represents data from a cohort of between 29 and 32 larvae; numbers above the bars are the numbers of foci characterized at each time point.

sible mechanism whereby secondary infection of host larvae could, in theory, be initiated prior to viral morphogenesis in primary cellular targets. More recently, Flipsen and coworkers (9) observed BV infection of midgut regenerative cells in *Spodoptera exigua* larvae before genome replication and late gene expression had occurred in the overlying, Ac*M*NPV ODV-infected midgut columnar cells. Finally, in our own studies of Ac*M*NPV-*hsp70*/*lacZ* pathogenesis in different host species (e.g., *H. virescens*, *T. ni*, *Manduca sexta*, *Spodoptera frugiperda*, and *Helicoverpa zea*), we have consistently observed differences of less than 4 h between the onset of early viral gene expression in ODV-infected midgut and BV-infected tracheal cells (7, 31, 34, 35; E. Haas-Stapleton, unpublished data). This time interval is far shorter than that required for de novo BV synthesis in cultured insect cells (~10 to 12 h). Thus, there is a growing body of empirical evidence that Ac*M*NPV, and perhaps other MNPVs, use early GP64 synthesis to repackage ODV-derived nucleocapsids as BV in order to accelerate systemic infection and circumvent the defensive sloughing response of their insect hosts.

During productive Ac*M*NPV infection, ODV midgut infections are eventually cleared completely after BV is transmitted to tracheal cells (34), leaving behind a viable midgut tissue. Transient infection of the midgut allows the host insect to

continue growing and to increase its biomass, providing more tissue that ultimately can become infected and used to generate millions of viral progeny. If Ac*M*NPV fails to establish a tracheal infection, however, the host survives and no progeny virus is released. Results from this study show that early synthesis of GP64 is one of two adaptive traits that enable a highly specialized infection strategy for avoiding the host's defensive response of sloughing. We previously showed that the other trait, packaging multiple nucleocapsids within ODV (i.e., the M trait), accelerated systemic infection relative to Ac*M*NPV ODV containing single nucleocapsids (36).

There are several described NPV species whose ODV contains only a single nucleocapsid (i.e., the "S" in SNPV [single-nucleocapsid NPV], such as *Helicoverpa zea* SNPV and *Heliothis armigera* SNPV). In addition to the Lepidoptera, SNPVs have been isolated from species in the Hymenoptera and Diptera (4). The host affinities of the SNPVs and the restriction of the MNPVs to the Lepidoptera, the most recently derived holometabolous insect order, suggest that the progenitor baculovirus was probably an SNPV. Phylogenetic evidence also supports the hypothesis that baculoviruses have evolved along with their insect hosts, rather than being derived from a recent invasion that spread across the insect orders (27). For Ac*M*NPV, the acquisition and early promotion of *gp64*, the development of two viral morphotypes and multiple nucleocapsid packaging of ODV, are all major evolutionary developments that contribute to its success as a pathogen of lepidopteran larvae.

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