Reduced Expression of the Immediate-Early Protein IE0 Enables Efficient Replication of *Autographa californica* Multiple Nucleopolyhedrovirus in Poorly Permissive *Spodoptera littoralis* Cells†

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Infection of *Spodoptera littoralis* SL2 cells with the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) results in apoptosis and low yields of viral progeny, in contrast to infection with *S. littoralis* nucleopolyhedrovirus (SINPV). By cotransfecting SL2 cells with AcMNPV genomic DNA and a cosmid library representing the complete SINPV genome, we were able to rescue AcMNPV replication and to isolate recombinant virus vAcSL2, which replicated efficiently in SL2 cells. Moreover, vAcSL2 showed enhanced infectivity for *S. littoralis* larvae compared to AcMNPV. The genome of vAcSL2 carried a 519-bp insert fragment that increased the distance between the TATA element and the transcriptional initiation site (CAGT) of immediate-early gene *ie0*. This finding correlated with low steady-state levels of IE0 and higher steady-state levels of IE1 (the product of the *ie1* gene, a major AcMNPV transactivator, and a multifunctional protein) than of IE0. Mutagenesis of the *ie0* promoter locus by insertion of the chloramphenicol acetyltransferase (*cat*) gene yielded a new recombinant AcMNPV with replication properties identical to those of vAcSL2. Thus, the analysis indicated that increasing the steady-state levels of IE1 relative to IE0 should enable AcMNPV replication in SL2 cells. This suggestion was confirmed by constructing a recombinant AcMNPV bearing an extra copy of the *ie1* gene under the control of the *Drosophila hsp70* promoter. These results suggest that IE0 plays a role in the regulation of AcMNPV infection and show, for the first time, that significant improvement in the ability of AcMNPV to replicate in a poorly permissive cell line and organism can be achieved by increasing the expression of the main multiple functional protein, IE1.

The arthropod virus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), a member of the *Baculoviridae* family, is able to infect 32 lepidopteran species (24). The complete genome of AcMNPV has been sequenced (3), and its replication in permissive cells has been studied extensively (7, 33). AcMNPV vectors are widely used as efficient tools for the expression of “foreign genes” in established cell lines and whole insects (7, 53). Recombinant AcMNPVs have been developed for use in pest control (29, 31, 48); moreover, baculovirus-based vectors have been considered for gene therapy purposes (1, 17). The ability of AcMNPV to interact with a wide range of hosts (50) makes it an attractive tool for elucidating which molecular factors are involved in determination of the host range of baculoviruses. Since AcMNPV can penetrate the nuclei of nonpermissive insect and mammalian cells, important mechanisms that determine the host range of AcMNPV are related to the expression of the viral genome (20, 44, 49–51).

AcMNPV gene expression is divided into early, late, and

very late phases. Early-phase genes are transcribed by host RNA polymerase II before DNA replication, while late- and very-late-phase genes depend on viral DNA replication and are transcribed by a virus-specific RNA polymerase (4, 27). AcMNPV protein IE1, the product of immediate-early gene *ie1*, plays a central role in regulating viral infection (8, 15, 22, 37, 41, 61). IE1 is a potent transcriptional activator of early genes, such as *39K*, *p35*, and *he65* (9, 22, 25, 39), and is essential for both DNA replication and late gene transcription (35, 37, 39, 62, 67). IE1 negatively regulates the transcription of the *pe38* and *ie2* genes (22, 38, 39). IE0, the product of the *ie0* gene, differs from IE1 only in that IE0 contains 54 additional amino acids at its amino terminus (resulting from splicing of the upstream *ie0* exon and its subsequent fusion to the *ie1* exon) (14). *ie0* RNA is expressed only during the early phase of infection, and *ie1* RNA is expressed during both early and late phases of infection (14, 36, 37). IE1 stimulates expression from the *ie1* promoter but down-regulates expression from the *ie0* promoter (37). The *ie0* gene product also transactivates the *ie1* promoter but does not affect expression from its own promoter (37).

The baculovirus genes *p143* (helicase), *hrf-1*, *hcf-1*, *p35*, and *ie2* were implicated in the determination of the host range of AcMNPV in various insect systems, including *Bombyx mori*, *Lymantria dispar*, *Trichoplusia ni*, and *Spodoptera frugiperda* (2, 13, 18, 44, 46, 56, 66).

Recently, it was reported that AcMNPV infection of SL2

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cells, a cell line derived from the Egyptian cotton worm *Spodoptera littoralis*, results in apoptosis and low yields of viral progeny (11, 19). The overexpression of *p35*, the apoptosis suppressor gene from AcMNPV (30), reduced apoptosis in AcMNPV-infected SL2 cells but did not improve the yield of AcMNPV budded viruses (BV) or viral infectivity for *S. littoralis* larvae (23). These results suggested that factors other than P35 were required by AcMNPV to replicate efficiently in SL2 cells. *S. littoralis* nucleopolyhedrovirus (SINPV) replicates successfully in SL2 cells. To study the possibility that SINPV could rescue AcMNPV infection, we cotransfected SL2 cells with AcMNPV DNA and a cosmid library representing the complete SINPV genome. We isolated vAcSL2, a recombinant AcMNPV that yielded high titers of viral progeny in SL2 cells. vAcSL2 carried a 519-bp insert that disrupted the *ie0* promoter, reducing the expression of this gene product and resulting in increased steady-state levels of IE1 relative to IE0. To confirm these observations, we mutagenized the *ie0* promoter locus by inserting the chloramphenicol acetyltransferase (*cat*) gene and obtained a new recombinant AcMNPV with replication properties identical to those of vAcSL2. These data suggested that the overexpression of *ie1* may enable efficient replication of AcMNPV in the SL2 host. Indeed, using various means to promote the overexpression of *ie1*, we were able to achieve productive AcMNPV infection of *S. littoralis* cells and larvae.

MATERIALS AND METHODS

Cell lines and viruses. *S. frugiperda* SF9 and *S. littoralis* SL2 cells were maintained and propagated in TNM-FH medium supplemented with 10% heat-inactivated fetal bovine serum (64). Wild-type AcMNPV strain E-2 (63) and SINPV strain E-15 (32) were propagated on SF9 cells. SINPV can replicate in both SF9 and SL2 cells. Viral growth curves were determined by plaque assays as described previously (23).

Plasmids and transfection. Plasmid pAcie1 contains an intact *ie1* gene (26). pHsp1 contains the *ie1* coding region under the control of the *Drosophila melanogaster* *hsp70* promoter. The latter was restricted from phsp70PLVI+CAT (16) by using *Xba*I and *Bam*HI and was subcloned into pBSK (Stratagene) bearing the *ie1* coding region *Hinc*II-*Hind*III DNA fragment from pAcie1. pBgl-11.1 and pBgl-3.3 bear 11.1- and 3.3-kbp *Bgl*II fragments of vAcSL2, respectively, each cloned at the *Bgl*II site of pBSK (see below); the 11.1-kbp fragment contains the *ie0* and *ie1* genes. pie0PCAT bears the *cat* gene inserted at AcMNPV nucleotide 122,800 (3) to disrupt the *ie0* promoter at position -31 upstream of the ATG start site. For this purpose, a 1.6-kbp *Sna*BI-*Sal*I fragment bearing the *ie0* promoter region was cloned into the corresponding site of pFastBac1 (Invitrogen Life Technologies). Then, a *Bam*HI site was created by mutagenesis with overlap extension PCR and with complementary primers containing the desired restriction site (55). The primers used were 5'-GCCGGATCCAGTAT AAGTAATTGA-3' and 5'-TTACTACTGGATCCGCGCGCGCA-3' (underlining indicates the *Bam*HI site). The *cat* gene from pQ39CAT (25) was digested with *Bam*HI and inserted into the newly created *Bam*HI site to yield pie0PCAT.

Generation of a recombinant baculovirus by homologous recombination in *Escherichia coli*. The polyhedrin (*polh*) gene, which the manufacturer deleted from AcMNPV BACmid (Invitrogen Life Technologies), was first reintroduced by site-specific transposition with transfer vector pFastBac1 into *E. coli* DH10BAC cells (45). For this purpose, the complete *polh* gene, including its own promoter and termination sequence, was rescued from pI1 (12) by digestion with *Xho*I, filling in with Klenow DNA polymerase, and further restriction with *Eco*RI and was inserted into *Eco*RI- and *Bst*1107I-digested pFastBac1 to obtain pFast-polh. Site-specific transposition with pFastpolh and selection of DH10BAC cells harboring recombinant BACmid AcFastpolh were performed by following the manufacturer's (Invitrogen Life Technologies) instructions. Then, to disrupt the *ie0* promoter in the viral (BACmid) genome, *E. coli* strain BJ5183 was transformed with recombinant BACmid AcFastpolh (5). The resulting strain, *E. coli* BJ-AcFP, was transformed with the 2.3-kb *Sna*BI-*Sal*I fragment containing the *cat* gene-disrupted *ie0* promoter sequence from pie0PCAT. Bacteria resistant to

kanamycin and chloramphenicol were selected, and high-molecular-weight mini-preparation DNA of recombinant BACmid AcFPCAT, with a *cat* gene insertion at the *ie0* promoter (Acie0PCAT), was prepared as described in the Bac-to-Bac protocol (Invitrogen Life Technologies). The presence of the *cat* gene insertion at the *ie0* promoter of BACmid AcFPCAT was confirmed by PCR with BACmid DNA template and primers CAT5' (5'-ATGGAGAAAAAACACTG-3'), CAT3' (5'-TTACGCCCGCCCT-3'), and IE0R (5'-GTGTCAACTTGCAAC TGCTGAGCTTCTGC-3'). Acie0PCAT DNA was used to transfect insect cells to obtain recombinant virus vAcie0PCAT.

DNA transfection of insect cells was performed with Lipofectin as described previously (19).

Rescue of AcMNPV replication in SL2 cells and isolation of AcMNPV recombinants. SL2 cells were cotransfected with either a complete cosmid library or individual cosmids of SINPV (19) or AcMNPV, respectively. At 96 h after transfection, the culture supernatants were removed and recombinant viruses that replicated and formed polyhedra in SL2 cells were isolated by subsequent plaque purification in these cells.

Restriction endonuclease and Southern blot analyses. Viral DNA prepared from BV (19) was digested with various restriction endonucleases and subjected to agarose gel electrophoresis (0.8% agarose) for 16 h at 30 V. After the run, the gel was quickly blotted to a nylon membrane (47). Hybridization was performed by using a GeneScreen Plus membrane according to the manufacturer's instructions (NEN Life Science Products). Probe labeling was performed by using a Renaissance random primer fluorescein labeling kit (NEN Life Science Products). The 519-bp insert fragment (IF) from vAcSL2 and the 2.4-kb *Eco*RV-*Hind*III fragment of the AcMNPV *ie1* gene (25) were used as hybridization probes.

Western blot analysis. Virus-infected or plasmid-transfected cells were harvested and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with a pH 9 separating gel; this procedure allowed us to distinguish clearly the IE0 molecular species from the IE1 molecular species. Immunoblot analysis was performed with anti-IE0/IE1 antiserum as previously described (23, 54).

Metabolic labeling of viral polypeptides. SF9 or SL2 cells (10^5 cells) were infected with either wild-type or recombinant AcMNPV. At 45 h postinfection, the cells were washed once with Sf900 II medium depleted of methionine and cysteine (Invitrogen Life Technologies). The medium was subsequently replaced with 500 μ l of the same medium supplemented with 15 μ Ci of a 35 S-labeled methionine-cysteine mixture (Amersham, Little Chalfont, United Kingdom). After 2 h, cell extracts were prepared by adding SDS lysis buffer (53) and then subjected to PAGE. Autoradiography was performed as described before (53).

Bioassays. Carefully selected third-instar *S. littoralis* larvae (24 per dose, in triplicate) were injected with various doses of AcMNPV or vAcSL2 BV. Control larvae were injected with the same volume of TNM-FH complete medium. The percent mortality was calculated as the number of dead larvae (excluding larvae killed by the injection, normally one or two) divided by the number of larvae that survived the infection. No mortality was observed for mock-infected larvae (12, 23).

An oral bioassay of third-instar *S. littoralis* was performed by using a diet containing six concentrations of wild-type and recombinant viral polyhedra ranging from 5×10^2 to 2.5×10^5 polyhedral inclusion bodies (PIB)/mm². After 24 h of exposure to the virus, the larvae were transferred to new containers containing a noncontaminated diet; larval death was monitored daily for 3 weeks. Thirty larvae were used for each dose, and the experiment was repeated twice.

RESULTS

Rescue of AcMNPV replication in *S. littoralis* SL2 cells. To study the potential of the SINPV genome to sustain the replication of AcMNPV in SL2 cells, we cotransfected the cells with AcMNPV DNA and an overlapping cosmid library representing the entire SINPV genome (19). After 6 days, we observed the appearance of polyhedra in the cell nuclei. By plaque purification, we were able to isolate a recombinant AcMNPV, vAcSL2, that replicated in SL2 and SF9 cells. At 96 h after infection of SL2 cells with vAcSL2, only 10 to 20% of the cells showed apoptosis, while 40 to 70% of the remaining cells showed clear polyhedra under direct microscopic observation (Fig. 1, upper left panel). In contrast, 90% of SL2 cells infected with AcMNPV at multiplicities of infection (MOIs) of 1 to 100

FIG. 1. vAcSL2 replicates in SL2 cells and forms polyhedra. SL2 and SF9 cells were infected with vAcSL2 or AcMNPV. Images are light micrographs (magnification, $\times 400$). Note the vAcSL2-infected cells producing polyhedra over the background of the intact cells, compared to AcMNPV-infected SL2 cell blebbing.

showed apoptosis and did not show PIB (Fig. 1, upper right panel). Compared to AcMNPV, vAcSL2 produced regular polyhedra in SF9 cells (Fig. 1, lower right and left panels, respectively). Since apoptosis has been implicated in the significant reduction of AcMNPV BV yields observed in SL2 cells (11), we compared the abilities of vAcSL2 and AcMNPV to replicate in these cells at MOIs of 0.5 and 5 (Fig. 2). BV were harvested from cell supernatants at 6, 9, 12, 28, and 48 h postinfection and titrated in SF9 cells. The titers of vAcSL2 obtained were significantly higher than those of AcMNPV: e.g., at 28 and 48 h after infection with an MOI of 0.5, the yield of vAcSL2 was 1,000-fold higher than that of AcMNPV (Fig. 2A).

Given that vAcSL2 replicated efficiently in SL2 cells, it was interesting to assess whether the vAcSL2 genome could rescue AcMNPV replication. For this purpose, we cotransfected SL2 cells with intact AcMNPV DNA and vAcSL2 DNA digested with various restriction enzymes. At about 72 to 96 h after transfection, the cells were examined microscopically for the presence of polyhedra. vAcSL2 DNA digested with either *Sma*I, *Xho*I, *Hind*III, or *Bgl*II rescued production of polyhedra, while *Eco*RV-digested DNA did not rescue it (Table 1). Remarkably, only *Eco*RV treatment is supposed to disrupt the *ie1* open reading frame. *Bgl*II-digested vAcSL2 genomic DNA showed two new fragments of about 11.1 and 3.3 kbp (designated α and β , respectively), not present in AcMNPV (Fig.

3A), due to the presence of a new *Bgl*II site in fragment D of *Bgl*II-digested AcMNPV (see below). To determine whether either of these two fragments was able to rescue AcMNPV amplification in SL2 cells, they were subcloned in pBSK to obtain plasmids pBgl-11.1 and pBgl-3.3 (see Materials and Methods). Transfection of SL2 cells with each of these plasmids and AcMNPV DNA showed that only pBgl-11.1 could rescue formation of polyhedra (Table 1).

Sequencing of the α and β fragments showed that the new *Bgl*II site corresponded to a point mutation in nucleotide 125 (A to G) in the coding region of viral gene *me53* which resulted in a conservative change (Lys 42 to Arg). The vAcSL2 11.1-kbp fragment that was able to rescue formation of polyhedra contained the whole *ie0-ie1* region of AcMNPV and included a new insert of 519 bp (named IF) located upstream of the *ie0* gene at position -88 from the ATG start site (Fig. 3B). The sequence 5'-TCGTAAATCAG-3' was repeated and flanked both ends of the IF at positions -78 to -88 and positions -586 to -597 from the *ie0* ATG start site (Fig. 3B, underlining). The IF increased the distance between the *ie0* CAGT transcriptional start site and the TATA element by 519 bp (Fig. 3B, bold underlining).

Expression of *ie0* in vAcSL2-infected cells. We observed that in AcMNPV-infected SL2 cells, the steady-state levels of IE1 and IE0 were almost identical throughout the infection (Fig.

FIG. 2. BV yields from vAcSL2- and AcMNPV-infected SL2 cells. BV were collected from clarified cell supernatants at various times after infection with vAcSL2 or AcMNPV and titrated in SF9 cells (in triplicate). Error bars show standard deviations.

4A; see also Fig. 6C); in contrast, in AcMNPV-infected permissive SF9 cells (Fig. 4B), the steady-state levels of IE1 early in infection were low relative to those of IE0 and later increased progressively. It should be noted that the double bands of IE1 and IE0 represented the phosphorylated and dephosphorylated main electrophoretic species reported (15) (see also below). The presence of the IF upstream of the transcriptional start site of *ie0* suggested that it might result in a lower level of expression of this gene in vAcSL2-infected cells. Indeed, IE0 was synthesized at very low steady-state levels in vAcSL2-infected SL2 and SF9 cells compared to AcMNPV-infected cells (Fig. 4A and B), and the steady-state levels of IE1 were higher than those of IE0.

A comparison of SF9 cells transfected with pBgl-11.1 and with pHspie1 (bearing only the *ie1* gene under the control of the *D. melanogaster hsp70* promoter) confirmed that IE1 was

the main electrophoretic species synthesized; IE1 was clearly distinguished from IE0 (15) (see also Materials and Methods) (Fig. 4C). IE0 was undetectable in pBgl-11.1-transfected cells; in comparison, AcMNPV-infected SF9 cells synthesized IE0 and IE1 (Fig. 4C).

To find out whether the IF of vAcSL2 was derived from the SINPV genome, we performed Southern blot analysis. SINPV, AcMNPV, and vAcSL2 genomic DNAs were digested with *Hind*III (Fig. 5A, lanes 2, 3, and 4, respectively) and *Pst*I (Fig. 5A, lanes 7, 8, and 9, respectively). Hybridization with labeled IF at 60°C showed that vAcSL2 bore the IF, as expected, but did not show fragments homologous to IF in the genomes of SINPV and AcMNPV (Fig. 5B, lanes 4 and 9, lanes 2 and 7, and lanes 3 and 8, respectively). Performing the hybridization at a lower stringency revealed a weak signal corresponding to the *Hind*III G and *Pst*I B fragments of AcMNPV (8.15 and 21.6 kbp, respectively) that bear the IE0 and IE1 coding sequences but not to SINPV. Also, Southern blot analysis with the same IF probe and cellular DNA did not indicate the presence of a homologous sequence in the SL2 cell genome (data not shown). A GenBank search did not reveal homology between the IF and any reported sequences. Thus, we concluded that the IF was probably the result of a recombinational event between AcMNPV and viral or cellular DNA, but we could not establish its exact genomic source.

To avoid the possibility that the phenotype of vAcSL2 was due to some unexpected mutation in the viral genome, we constructed a recombinant AcMNPV in which the *ie0* promoter region was disrupted by insertion of the *cat* gene. For this purpose, we used the method described by Bideshi and Federici (5). Plasmid pie0PCAT was used to transpose the

TABLE 1. Rescue of wild-type AcMNPV replication in SL2 cells by vAcSL2 DNA fragments^a

DNA transfected	Polyhedra
AcMNPV	---
vAcSL2	+++
AcMNPV + <i>Sma</i> I-digested vAcSL2	++
AcMNPV + <i>Hind</i> III-digested vAcSL2	++
AcMNPV + <i>Xho</i> I-digested vAcSL2	++
AcMNPV + <i>Eco</i> RV-digested vAcSL2	---
AcMNPV + pBSK	---
AcMNPV + pBgl-11.1	++

^a SL2 cells (10⁵) were transfected with the indicated DNAs (1 μg) and examined 3 to 4 days later with a light microscope for the appearance of viral polyhedra. +++ and ++, 30 and 20%, respectively, of cells exhibited polyhedra; ---, no polyhedra were evident.

FIG. 3. vAcSL2 contains a 519-bp insert downstream of the *ie0* TATA element that disrupts the basic transcriptional unit. (A) vAcSL2 DNA (lane 1) and AcMNPV DNA (lane 2) were digested with *Bgl*II, and the restricted DNA fragments were analyzed in a 0.7% agarose gel. New *Bgl*II fragments in vAcSL2 are indicated by arrows (α and β). Lane M, molecular size markers (λ DNA digested with *Bst*EII) (in kilobase pairs). (B) Partial physical map and sequence of fragment α downstream of the new *Bgl*II site. Indicated are the *ie0* TATA element (bold), the transcriptional initiation motif CAGT (bold and underlined), and the downstream IF sequence that increases the distance between the motifs (repeated sequence is underlined). AcMNPV map units are indicated as well. The boxes show the locations of the *ie0* 5'-terminal coding region and the *me53* gene, which are transcribed in opposite directions (arrows). Restriction sites: H, *Hind*III; B, *Bgl*II; S, *Sal*I.

mutated *ie0* promoter region into BACmid AcFastpolh, and the resultant BACmid construct was transfected into SF9 cells to further isolate recombinant baculovirus vAcie0PCAT (see Materials and Methods) (Fig. 6A). The constructs were confirmed by PCR analysis with a primer complementary to the *cat* gene and a primer 159 bp downstream of the transposition site (CAT3' and IE0R, respectively) (Fig. 6B). The effect of disruption of the *ie0* promoter on the synthesis of IE0 and IE1 during the infectious cycle of vAcie0PCAT was studied as described above by using immunoblot analysis (Fig. 6C). vAcie0PCAT infection of SL2 cells reproduced the patterns of expression of *ie0* and *ie1* observed with vAcSL2 expressing mainly *ie1* (compare to AcMNPV-infected cells and to pHspie1-transfected cells in Fig. 6C and Fig. 4A). Direct microscopic observation of vAcie0PCAT-infected SL2 cells re-

vealed polyhedra (data not shown). Moreover, vAcie0PCAT showed a significant improvement in BV yields in SL2 cells compared to wild-type AcMNPV (Fig. 7). Identical results were obtained when the *cat* gene was inserted in the opposite orientation (data not shown).

Formation of AcMNPV polyhedra can be rescued in SL2 cells by increasing the level of expression of *ie1*. The above results suggested that enhancing *ie1* expression might rescue the replication of AcMNPV in SL2 cells. To test this hypothesis, we cotransfected SL2 cells with either pBgl-11.1 or pHspie1 and AcMNPV DNA. At 72 to 96 h after transfection, formation of polyhedra was detected in the transfected cells, while control cells cotransfected with pBSK and AcMNPV DNA did not show polyhedra (data not shown). Moreover, by subsequent plaque purification, we were able to isolate from

FIG. 4. IE1 and IE0 synthesis in vAcSL2- and AcMNPV-infected cells. (A and B) SL2 and SF9 cells (10^5) were infected with vAcSL2 and AcMNPV at an MOI of 5. At various times postinfection, the cells were harvested, and cell extracts were subjected to SDS-PAGE and immunoblot analysis with anti-IE0/IE1 antiserum. mi, mock-infected cells. (C) Expression of *ie1* in transfected cells. SF9 cells (10^5) were transfected with 1 μ g of pBgl-11.1 (bearing the α fragment) or pHspie1 (bearing *ie1* under the control of the *hsp70* promoter). At 24 h after transfection, cell extracts were subjected to SDS-PAGE and immunoblot analysis. m-transf., mock-transfected cells. Molecular mass markers are indicated.

pHspie1 and pBgl-11.1 cotransfection supernatants new recombinant viruses (vHsp-1 and vBgl3, respectively) that replicated in SL2 cells, forming polyhedra. Southern blot analysis with an *ie1* probe (see Materials and Methods) showed that vHsp-1 bore two copies of the *ie1* gene (Fig. 5C, lanes 6 and 11). vBgl3 bore the IF in the viral genome (Fig. 5C, lanes 5 and 10). Lighter bands observed in all of the lanes corresponded to the common background usually observed with this chemiluminescence detection method under these exposure conditions (Fig. 5C). Thus, in the first case, cotransfection with pHspie1 appeared to have resulted in the insertion of a second *ie1* gene in the viral genome. In the second case, the region upstream of the *ie0* gene appeared to have been replaced by an IF copy.

Infection of SF9 cells with vAcSL2, vHsp-1, vBgl3, and AcMNPV followed by metabolic labeling with 35 S-labeled amino acids (see Materials and Methods) resulted in similar polypeptide profiles (Fig. 8, lanes 3, 4, 5, and 2, respectively). The most prominent polypeptide detected at 45 h postinfection was polyhedrin. This marker, which reflected the successful completion of the viral replication cycle, was clearly detected in SL2 cells infected with vAcSL2, vBgl3, and vHsp-1 but not AcMNPV (Fig. 8, lanes 8, 9, 10, and 7, respectively). As expected, polyhedrin was not observed in mock-infected SF9 and SL2 cells (Fig. 8, lanes 1 and 6, respectively).

The expression of *ie1* was implicated in the induction of apoptosis in *S. frugiperda* cells (57). Also, it was reported that AcMNPV-infected SL2 cells underwent apoptosis and expressed the antiapoptotic gene *p35* at very low levels (23). Thus, we hypothesized that the overexpression of *ie1* would enhance *p35* expression as well, neutralizing the potential increase in the apoptotic response of the cell. Indeed, P35 steady-state levels were higher in vAcSL2-infected cells than in

AcMNPV-infected SL2 cells (Fig. 9, lanes 1 to 3 and lanes 4 to 6, respectively).

Infectivity of vAcSL2 for *S. littoralis* larvae. To determine whether the ability of vAcSL2 to replicate efficiently in SL2 cells is a cell line phenomenon or has organismic implications, we compared the infectivity of vAcSL2 with that of AcMNPV for *S. littoralis* larvae. For this purpose, *S. littoralis* third-instar larvae were infected with increasing doses of viral polyhedra orally (see Materials and Methods) or via the intrahemocoelic route (by injection of increasing doses of BV). No differences in AcMNPV and vAcSL2 infectivities were measured for the oral route which, under these conditions, resulted in only 10 to 14% larval death at the higher PIB doses (data not shown). However, significant differences in larval mortality were observed for intrahemocoelic inoculation of BV: a 10-fold increase in the AcMNPV dose was required to obtain 50 and 70% mortality of the larval population (at doses of 100 and $\sim 1,000$ PFU, respectively) (Fig. 10).

DISCUSSION

Expression of *ie1* and replication of AcMNPV in *S. littoralis*. IE1 displayed steady-state levels equivalent to or lower than those of IE0 during the early phase of AcMNPV infection of permissive SF9 cells (Fig. 4B and 6C). Late in infection, IE1 steady-state levels increased significantly over those of IE0 in SF9 cells (54 and *ibid*), but in poorly permissive SL2 cells, the steady-state levels of IE1 and IE0 remained equivalent throughout the infection (Fig. 4A and 6C). The IF disrupted the continuity of the *ie0* transcriptional unit by increasing the distance between the TATA element and the CAGT transcriptional start site (6, 22, 58), suggesting that indeed the transcrip-

FIG. 5. Southern blot analysis of AcMNPV recombinants. (A) *Hin*-*III*- or *Pst**I*-digested viral DNAs. (B) Hybridization with fluorescein-labeled IF (PCR amplified from pBgl-11.1) at 60°C. (C) Hybridization with fluorescein-labeled *EcoRV-Hin**III* *ie1* fragment. Lanes 2 and 7, SINPV; lanes 3 and 8, AcMNPV; lanes 4 and 9, vAcSL2; lanes 5 and 10, vBgl3; lanes 6 and 11, vHsp-1; lane 1, undigested pBgl-11.1; lane M, molecular size markers (in kilobase pairs).

4), resulting in higher steady-state levels of IE1 than of IE0 in vAcSL2-infected SL2 cells compared to AcMNPV-infected cells (Fig. 4A). vAcSL2 showed a significant increase in infectivity toward *S. littoralis* SL2 cells (Fig. 1) and larvae (Fig. 10, BV injection). However, no change in infectivity was measured when infection was performed via the oral route. Thus, it appears that viral factors besides the overexpression of IE1 relative to IE0 were required to overcome the initial response of *S. littoralis* larvae (probably of immune origin) (68) to viral infection.

The IF displayed the same repeat sequence, 5'-TCGTAAA TCAG-3', at its 5' and 3' termini, suggesting that it originated by some recombinational event between viral (AcMNPV or SINPV) and cellular (SL2) sequences. However, we could not determine the exact origin of the IF by Southern blot analysis. Thus, we evaluated whether the insertion of another arbitrary DNA fragment (e.g., the *cat* gene) (Fig. 6) instead of the IF into the *ie0* promoter locus might reproduce the vAcSL2 phenotype. Indeed, *cat* gene-mediated disruption of the *ie0* promoter region resulted in enhanced replication of recombinant vAcie0PCAT in SL2 cells (Fig. 7) that correlated with lower steady-state levels of IE0 than of IE1 (Fig. 6C).

The above data suggested the following. (i) There was a block to *ie1* expression in AcMNPV-infected SL2 cells (probably late expression, although it was not rigorously measured

tion of *ie0* would be diminished in vAcSL2-infected cells; however, this aspect was not directly analyzed in this study (e.g., by transcriptional mapping). However, the steady-state levels of IE0 were reduced consistently in vAcSL2-infected cells (Fig.

FIG. 6. vAcie0PCAT structure and analysis and synthesis of IE1 and IE0. (A) Genomic organization. The dashed lines indicate the site of insertion of the *cat* gene (box). The other boxes show the locations of the *ie0* coding region (exon 1 and exon 2) and the *ie1* and *me53* genes and their corresponding promoters (upper arrows). IE0R, CAT5', and CAT3' were primers used for PCR analysis. The dotted lines indicate the expected sizes of amplified fragments. (B) PCR analysis. (Left panel) *cat* gene amplification. Lane 1, no template; lane 2, AcMNPV; lane 3, vAcie0PCAT. (Right panel) Lane 1, *cat* gene marker (same as lane 3 in the left panel); lanes 2 to 4, *cat* gene plus exon 1 (primers CAT3' and IE0R), with no template in lane 2, AcMNPV in lane 3, and vAcie0PCAT in lane 4. (C) SL2 (10^5 cells) were infected with AcMNPV and vAcie0PCAT at an MOI of 5. pHspie1, SF9-transfected cell control. At various times postinfection, cell extracts were subjected to SDS-PAGE and immunoblot analysis with anti-IE0/IE1 antiserum as described in the legend to Fig. 4. Molecular mass markers are indicated.

by comparing it with *ie1* expression in aphidicolin-treated infected cells) (compare Fig. 4A and B). (ii) An increase in the steady-state levels of IE1 relative to IE0 may enable the replication of AcMNPV in SL2 cells (see below).

We confirmed the latter hypothesis by cotransfecting AcMNPV and plasmid pHspie1 or the *ie0-ie1* genetic unit present in the vAcSL2 genome (pBgl-11.1) and subsequently isolating new recombinant viruses that overexpressed *ie1* (Table 1 and

FIG. 7. BV yields from vAcieOPCAT- and AcMNPV-infected SL2 cells. BV were collected from clarified cell supernatants at various times after infection (p.i.) with an MOI of 0.5 and titrated in SF9 cells (in triplicate). Error bars indicate standard deviations.

Fig. 8). Our results provide the first evidence that AcMNPV can replicate in a poorly permissive cell line (and insect) through an increase in the level of expression of the *ie1* gene (see below).

IE0 and IE1 functions. IE1 is a multifunctional protein that plays a crucial role in the life cycle of AcMNPV: (i) transactivation of the delayed early and late classes of genes (37, 39), (ii) its own continued expression during infection, and (iii) repression of other immediate-early genes.

FIG. 8. Metabolic labeling of polypeptides synthesized in virus-infected SF9 and SL2 cells. Cells (10^5) were infected at an MOI of 20. At 45 h postinfection, metabolic labeling was performed with a ^{35}S -labeled methionine-cysteine mixture. Analysis was done with extracts from cells infected with AcMNPV (lanes 2 and 7), vAcSL2 (lanes 3 and 8), vBgl3 (lanes 4 and 9), and vHsp-1 (lanes 5 and 10) and with mock-infected SF9 cells (lane 1) and SL2 cells (lane 6). Ph, location of AcMNPV polyhedrin.

FIG. 9. Synthesis of the apoptosis suppressor P35 in vAcSL2-infected SL2 cells. SL2 cells were infected at an MOI of 5 with vAcSL2 (lanes 1 to 3) or AcMNPV (lanes 4 to 6). Cell extracts (10^5 cell equivalents) were harvested at various times postinfection and subjected to SDS-PAGE and immunoblot analysis with anti-P35 antiserum (23). A molecular mass marker is indicated.

The finding that lower steady-state levels of IE0 and higher steady-state levels of IE1 enabled AcMNPV replication in SL2 cells suggested that IE0 may be involved in the regulation of IE1 function. Thus, elevated IE1 levels may be required in SL2 cells to stimulate genes involved in AcMNPV DNA replication, such as DNA polymerase, *p35*, and helicase genes (10, 43, 52, 54, 67), to become directly involved in binding to a replication

FIG. 10. Mortality of *S. littoralis* larvae infected with vAcSL2 and AcMNPV. *S. littoralis* larvae (third instar, 24 larvae per dose) were injected with increasing concentrations of AcMNPV or vAcSL2 BV. The mortality rate was calculated as the number of larvae that died from the infection divided by the number of survivors. The results are the averages of three independent experiments. Error bars indicate standard deviations.

origin and catalyzing early steps that lead to the assembly of a replication complex (33, 42). Also, the reduction of apoptosis in vAcSL2-infected SL2 cells may be the result of enhanced *p35* gene expression (Fig. 9) (23).

IE1 has separate domains for transactivation and DNA binding that are essential for the modulation of baculovirus gene expression (35, 60, 61). Little is known about the role of IE0 as a transregulator, although it possesses an additional N-terminal 54 amino acids compared to IE1. Transient expression assays revealed that IE0 could transactivate the *ie1* promoter but did not affect expression from the *ie0* promoter, while IE1 stimulated expression from the *ie1* promoter but down-regulated expression from the *ie0* promoter (37). IE1 was shown to bind, probably as a dimer (60), to specific DNA sequences present in *hrs* that have been demonstrated to function as *cis*-acting enhancers of viral transcription and origins of replication (34, 40, 41, 59) and to sequences present in promoters of genes that it down-regulates (41). The motif 5'-ACBYGTAA-3' is present in all DNA fragments that have been shown to bind IE1, including *ie0* (41). Thus, it was proposed that transcriptional repression by IE1 occurs when such a single binding motif is present in the DNA sequence and that IE1 may have a lower affinity for these single motifs than for the complete palindromic sequences present in the *hrs* (42).

The same IE1 domains that are involved in DNA binding are present in IE0. Moreover, it was suggested that the putative helix-loop-helix domain present at C-terminal residues 543 to 568 mediates oligomerization and DNA binding of IE1 (61). Taken together, these and the above data suggest that IE0 and IE1 can form heterodimers (39). It is conceivable that the IE0-IE1 heterodimer has a function different from that of the IE1-IE1 homodimer: IE1 or the IE1-IE1 dimer could up-regulate the *ie1* promoter and down-regulate the *ie0* promoter (41), but the formation of an IE0-IE1 complex could compete for free IE1 molecules, preventing the formation of IE1-IE1 dimers, and/or could compete for DNA binding of the IE1 homodimer, which is required to enhance the expression of *ie1* and other IE1-transactivatable promoters.

In addition, it was postulated that a host factor(s) which transactivates the *Orgyia pseudotsugata* multicapsid nucleopolyhedrovirus (OpMNPV) *ie1* promoter is present in SF9 cells but absent in Ld652Y cells (21), and a 38-kDa protein with *hr* gene binding capacity was isolated from SF9 cells (28). Similarly, such a factor could be missing or not very abundant in SL2 cells, resulting in a low level of *ie1* expression late in infection and consequently in an insufficient amount of functional IE1 to compete with the IE1-IE0 complex or to displace IE0 from it and transactivate essential AcMNPV replication genes. Either the reduction of *ie0* expression or the overexpression of *ie1* could overcome the IE1 deficiency. A variation of this model could include cellular proteins in the IE0-IE1 complex.

In support of the IE0-IE1 dimer hypothesis, a recent study suggested that IE0 and IE1 interact in regulating the expression of the AcMNPV *he65* gene during the infectious cycle (39). Moreover, IE0 was postulated to be part of a tripartite protein-DNA-IE1 complex (39, 60). Recently, it was observed that a gradation of gene activation occurs in transfected Ld652Y cells, depending on the presence of IE1 alone, IE1 and IE0, or IE0 alone from OpMNPV (65). It was proposed

that maintaining a low level of IE0 is a priority during a normal OpMNPV infection (65). Further studies will be required to prove directly the IE1-IE0 protein-protein interaction hypothesis and its biological role.

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