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Baculovirus-Mediated Expression of Bacterial Genes in Dipteran and Mammalian Cells†

LUIS F. CARBONELL,[1] MARC J. KLOWDEN,[2] AND LOIS K. MILLER[1]*

Department of Bacteriology and Biochemistry[1] and Department of Plant, Soil and Entomological Sciences,[2] University of Idaho, Moscow, Idaho 83843

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A recombinant baculovirus containing the *Escherichia coli* chloramphenicol acetyltransferase (CAT) gene under the control of the Rous sarcoma virus long terminal repeat promoter and the *E. coli* β-galactosidase gene under the control of the very late baculoviral polyhedrin promoter was used to determine if *Autographa californica* nuclear polyhedrosis virus, a baculovirus of *Lepidoptera*, can enter and express viral DNA in dipteran (*Drosophila* sp.) and mammalian (*Mus* sp.) cells that are considered refractory to baculovirus replication. Following infection, CAT gene expression was observed in both dipteran and mammalian cells, but expression in the mammalian cell line was less than 0.05% of that observed in either dipteran or lepidopteran cells. Although the level of CAT gene expression was similar in permissive lepidopteran and nonpermissive dipteran cells, expression of β-galactosidase activity from the late polyhedrin promoter in dipteran or mammalian cells was less than 0.3% of the levels observed in lepidopteran cells. These results indicate that foreign gene expression in nonpermissive cells is promoter dependent and that late viral gene expression is restricted in these cells. The Rous sarcoma virus long terminal repeat allows substantial CAT gene expression in both a *D. melanogaster* cell line and *Aedes aegypti* midgut cells. Baculovirus DNA undergoes a limited number of replications in *Drosophila* cells. The results are relevant to baculovirus host range, the safety of baculoviruses as pesticides, and the development of baculovirus pesticides with expanded host ranges.

Because of the interest in using insect baculoviruses as biological pesticides (3), the host specificity of these viruses is important with regard to both their applicability and their safety. From an ecological perspective, a pesticide with a narrow host specificity is considered desirable because it provides a wide margin of safety for nontarget organisms. From the perspective of commercial production and marketing, however, the ability of a single baculovirus to kill only a very limited number of insect species is viewed as economically disadvantageous because of the need for large numbers of different products with limited applicability. Currently there is industrial interest in expanding the host ranges of commercially produced baculoviruses by using recombinant DNA technology.

The host specificity of baculoviruses has been a subject of considerable discussion owing to safety considerations with regard to their use as pesticides (32, 33). Very little, however, is known about the factors that control the host specificity of baculoviruses. *Autographa californica* nuclear polyhedrosis virus (AcNPV) is considered to have a relatively broad host range for a baculovirus and is known to infect over 30 different species of *Lepidoptera*. Entry of AcNPV into vertebrate cells is well documented (10, 21, 35, 36). Although replication of AcNPV in dipteran and mammalian cells has been reported (13, 22, 30), the experimental methods and interpretations have been questioned by others (12). Following infection, at least some of the viral DNA reaches the mammalian nucleus, but it does not persist, and there is no evidence that baculovirus DNA is transcribed in mammalian cells (35). Gene expression was not detected after infection of either a dipteran or a mammalian cell line with a recombinant AcNPV containing an *Escherichia coli* β-galactosidase gene fusion under the control of a strong late promoter, the polyhedrin promoter. The β-galactosidase assay was sufficiently sensitive to approximately 0.1% of the activity observed in permissive lepidopteran cells infected with the recombinant AcNPV (28).

Using a recombinant AcNPV containing the *E. coli* gene encoding chloramphenicol acetyltransferase (CAT) under the control of an exogenous promoter, the long terminal repeat (LTR) of Rous sarcoma virus (RSV), we now show that a gene carried as part of the DNA genome of a lepidopteran-specific baculovirus is abundantly expressed in dipteran cells if gene transcription is controlled by a promoter which is active in uninfected cells. The baculovirus can also enter and express genes in mammalian cells, but much less efficiently. The level and temporal regulation of expression of foreign genes under the control of the two promoters in both permissive and nonpermissive cells is described. The results are discussed with respect to determining factors affecting the host ranges of viruses, expanding the effective host ranges of genetically engineered viral pesticides, and the resulting safety of such recombinant DNA-derived pesticides.

MATERIALS AND METHODS

Cells and virus. All cell lines and the media used were described previously (28). All viruses used as inocula were propagated in the *Spodoptera frugiperda* IPLB-SF-21 cell line. The wild-type virus was the L-1 variant of AcNPV (18, 27). A recombinant AcNPV L1GP-gal3 containing a polyhedrin–β-galactosidase fusion gene under the control of the polyhedrin promoter was described previously (28). A new recombinant virus, AcNPV L1LC-galcat was constructed by allelic replacement (marker rescue) of the polyhedrin gene of AcNPV L-1 with DNA sequences from the plasmid pLC-1 (Fig. 1). The procedure for allelic replacement with pLC-1 was similar to that described for AcNPV

* Corresponding author.
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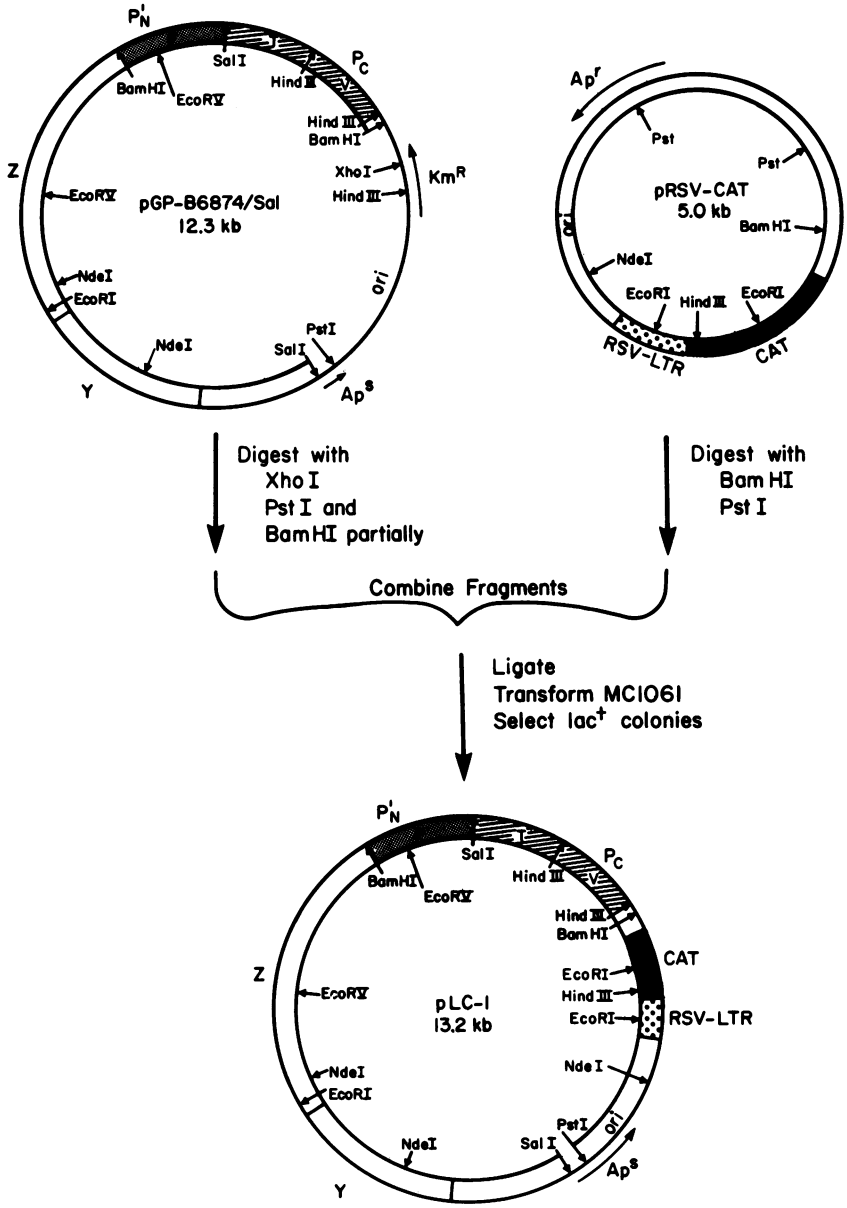

FIG. 1. Construction of plasmid pLC-1. We constructed plasmid pLC-1 by removing a section of pGPB6874/Sal (28) containing an origin for replication in *E. coli* and Km^r by using *Pst*I and *Bam*HI and replacing that section with a *Pst*I-*Bam*HI fragment of pRSVcat (9) containing a portion of the β-lactamase gene (Ap^s), an origin of replication, the RSV LTR acting as a promoter for the CAT gene, and a polyadenylation site from simian virus 40. *E. coli* MC1061 was transformed with the ligation products, and pLC-1 was selected by growth on minimal agar plates containing lactose.

L1GP-gal3 construction (28). Briefly, wild-type virus and plasmid DNAs were cotransfected into *S. frugiperda* cells, the cells were overlaid with complete TC100 medium (GIBCO Laboratories, Grand Island, N.Y.) containing agarose and a β-galactosidase indicator, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Plaques of recombinant viruses were selected by their blue color in the agarose overlay, and each plaque was directly replaque purified on *S. frugiperda* cells. The second plaque purification involved a selection for plaques with a blue color but lacking polyhedra (occlusion bodies) in the infected centers. Blue, polyhedra-negative plaques were selected, virus stocks were produced, and the viral DNA was screened by restriction endonuclease analysis with *Bam*HI, *Pst*I, *Hin*dIII, and *Eco*RI to ensure that the recombinant virus contained all of the sequences of pLC-1 between the polyhedrin amino and carboxyl termini and lacked an intact polyhedrin gene.

Construction of pLC-1. The plasmid pLC-1 was constructed according to the general scheme outlined in Fig. 1. Construction of the starting plasmids, pCPB6874/Sal and pRSVcat, was described previously (9, 28). Plasmid pGPB6874/Sal DNA was digested with *Xho*I and *Pst*I to completion and with *Bam*HI partially so that the small *Pst*I-*Bam*HI fragment containing an origin of replication in *E. coli* and the Km^r gene would be detached from the bulk of the plasmid DNA and further broken at the *Xho*I site. This *Pst*I-*Bam*HI region of pGPB6874/Sal was replaced with a *Pst*I-*Bam*HI fragment from pRSVcat containing an *E. coli*

replication origin (ori), the CAT gene under the control of the RSV LTR promoter, and a portion of the β-lactamase gene (Ap^s). After ligation with T4 DNA ligase (20) and transformation into *E. coli* MC1061 [Δ(*lac*IPOZYA)74 *galU galK* $strA^r$ $hsdR^-$ Δ(*ara leu*)] (4), the appropriate ligation product, pLC-1, was selected on minimal agar plates (24) containing 0.2% lactose, 40 μg of leucine per ml, and 50 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per ml, thus selecting for lactose-utilizing colonies. Further tests also showed that pLC-1 conferred resistance to chloramphenicol, but this property was not used in the original selection for pLC-1. The physical nature of pLC-1 DNA was confirmed by restriction endonuclease analysis with *Bam*HI, *Pst*I, *Sal*I, *Hin*dIII, and *Eco*RI.

Enzyme and protein assays. Cells were washed with phosphate-buffered saline (19) and scraped from plates with a rubber policeman in 1 ml of phosphate-buffered saline. The cells were pelleted at 200 × *g* for 10 min in Eppendorf tubes, washed with 1 ml of phosphate-buffered saline, frozen in liquid N_2, and stored at −20°C until assayed. For the assays, cells were suspended in 0.25 M Tris hydrochloride (pH 7.8) and disrupted by freeze-thawing in dry ice-ethanol three times at 5-min intervals. The cell debris was pelleted in an Eppendorf Microfuge (Beckman Instruments, Inc., Fullerton, Calif.) for 10 min, and the supernatant was assayed for β-galactosidase (24, 28) and CAT activities (9). One hundred nCi (2 nmol) of [^{14}C]chloramphenicol (New England Nuclear Corp., Boston, Mass.) was used per CAT assay. We quantitated the amount of acetylated material by excising the regions of the thin-layer support and counting the radioisotope by liquid scintillation. For accurate quantitation of CAT levels, cell extracts were diluted so that 30% or less of the input chloramphenicol was acetylated. β-Galactosidase levels are reported in Miller units (nanomoles of *o*-nitophenyl-β-D-galactopyranoside cleaved per min per mg of protein) (24), and CAT levels are reported as nanomoles of chloramphenicol acetylated per min per mg of protein. Protein was quantitated by a protein assay (Bio-Rad Laboratories, Richmond, Calif.) based on the Bradford method (1).

Infection of mosquito midguts. Virus was introduced into the midguts of adult female *Aedes aegypti* UGAL 3 days postemergence via the anus with a micropipette (16). Thirty females were used per virus inoculum; each female received 4 μl of the virus preparation. The AcNPV L1LC-galcat preparation was 4.9×10^9 PFU/ml (blue plaques on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside plates), and AcNPV L-1 was 8.0×10^9 PFU/ml; the virus inocula were suspended in *Drosophila* tissue culture medium (28).

Inoculated mosquitoes were maintained at 27°C on a 10% sucrose diet in double containment cages especially designed for retaining recombinant DNA virus-infected mosquitoes. At 48 h postenema, the mosquitoes were chilled to 4°C, and the midguts were removed by dissection and placed in 50 μl of 0.25 M Tris hydrochloride (pH 7.8). Extracts were made from 28 midguts of AcNPV L-1-inoculated females and 28 midguts of AcNPV L1LC-galcat-inoculated females. The extracts were prepared and assayed for β-galactosidase, CAT, and protein as described above.

Infection of mammalian cells. Virus inoculum was grown in *S. frugiperda* cells, pelleted, and suspended in RPMI-1640 medium (GIBCO) containing 13% fetal bovine serum (KC Biologicals, Lenexa, Kans.) and antibiotics. Monolayers of mouse L929 cells (29) were infected at a multiplicity of infection (MOI) of 800 at room temperature for 1 h. In some cases, the inoculum was removed after the 1-h adsorption period. Fresh medium was added, and the cells were incubated at 37°C in an atmosphere containing 5% CO_2. Cells were collected at 12, 24, 48, and 96 h postinfection (p.i.), washed with phosphate-buffered saline (19), pelleted, and frozen in liquid N_2. Enzyme assays were performed as described above.

Incorporation of [^{3}H]thymidine. Monolayers of cells were preincubated for 2 h in medium with or without 50 μg of cytosine arabinoside (ara-C) per ml, infected with AcNPV L1LC-galcat at an MOI of 20, and pulse-labeled for 3 h with appropriate medium containing 30 μCi of [^{3}H]thymidine (78.0 μCi/mmol) (New England Nuclear) per ml at various times after infection. Incorporation was stopped by adding 15 mM NaN_3 and 1 mM KCN. Cells were scraped from the plate and centrifuged along with medium in an Eppendorf Microfuge for 30 s. Pellets were suspended in 10 mM Tris hydrochloride (pH 7.8)–10 mM EDTA–0.25% sodium dodecyl sulfate–300 μg of proteinase K per ml and incubated at 37°C for 12 h. Samples were spotted onto GF/C glass microfiber filters (Whatman, Inc., Clifton, N.J.) prewet with 20% trichloroacetic acid, washed with 10% trichloroacetic acid, and then washed with ethanol. Acid-precipitable radioactivity was determined by scintillation counting.

Dot blot hybridization. Monolayers of cells were preincubated for 2 h with or without ara-C, inoculated at an MOI of 5 for *S. frugiperda* cells (50 μg of ara-C per ml) or an MOI of 5 and 50 for *D. melanogaster* cells (100 μg of ara-C per ml) with gentle rocking for 1 h at room temperature, and then incubated with fresh medium with or without ara-C at 27°C. The procedure for cell and virus collection, DNA isolation, and dot blotting has been described previously (27). The filter was probed with ^{32}P-labeled pLC-1 DNA prepared by nick translation (20). The amount of DNA hybridized in each dot was quantitated by inclusion of known quantities of L1LC-galcat DNA in separate dots. The dots were individually cut from the blots after autoradiography, and radioactivity was determined by scintillation counting. Standard curves were prepared from the L1LC-galcat DNA dilutions; DNA levels in the other dots were quantitated in relationship to the standard curve. In the case of DNA derived from infected *S. frugiperda* cells, it was necessary to dilute the DNA samples applied to dots for more accurate quantitation.

RESULTS

Regulation of expression of AcNPV L1LC-galcat-borne genes in permissive lepidopteran cells. A recombinant virus, AcNPV L1LC-galcat, was constructed by allelic replacement of the nonessential wild-type polyhedrin gene with pLC-1 sequences (Fig. 1; see above). AcNPV L1LC-galcat thus carries an approximately 10-kilobase insert containing, in order, the polyhedrin promoter, the polyhedrin N terminus fused in-frame with the *E. coli* β-galactosidase gene, the *lacY* gene, part of the *lacA* gene, a portion of the β-lactamase gene, a *colE1* origin for replication in *E. coli*, the RSV LTR promoter, the CAT gene, and a polyadenylation signal from simian virus 40 DNA followed by the C-terminal regions of polyhedrin.

We determined the temporal patterns of expression of the polyhedrin–β-galactosidase fusion and CAT genes after inoculation of the permissive *S. frugiperda* IPLB-SF-21 cell line with AcNPV L1LC-galcat by assaying the enzymatic activities at various times p.i. (Fig. 2). As expected from previous studies with AcNPV L1GP-gal3, the polyhedrin–β-galactosidase fusion gene under control of the polyhedrin promoter was expressed as an occlusion-specific protein; β-galactosidase activity was not detected through 12 h p.i.,

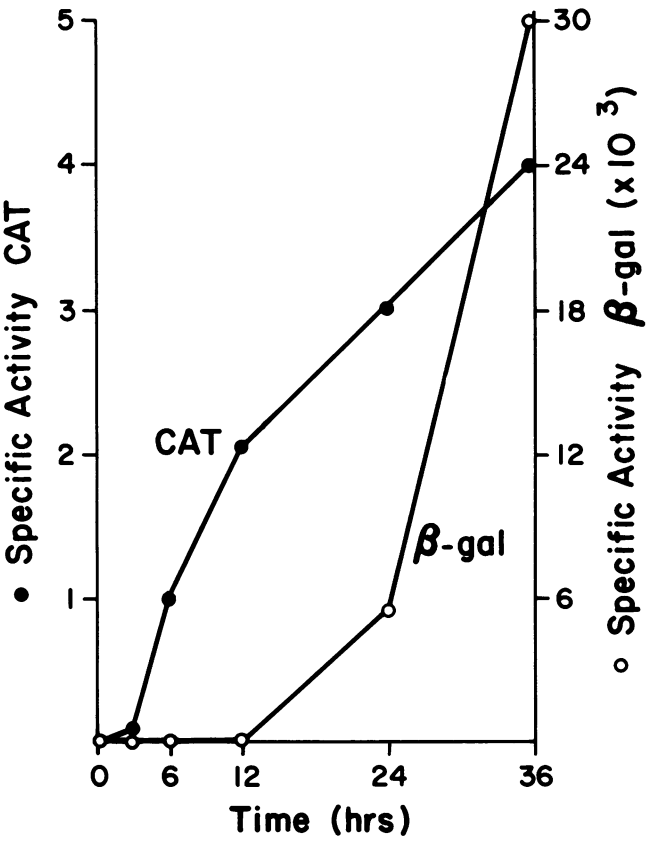

FIG. 2. Expression of CAT and β-galactosidase in AcNPV L1LC-galcat-infected *S. frugiperda* cells. *S. frugiperda* cells were infected with AcNPV L1LC-galcat at an MOI of 20. At various times p.i., cells were washed, pelleted, and assayed for CAT, β-galactosidase, and protein.

but it was detected by 24 h p.i. Activity increased dramatically from 24 through 36 h p.i. and represented an approximately 1,000-fold increase above the background levels observed at 12 h p.i. In contrast, CAT activity was first observed at 2 h p.i., increased substantially through 12 h p.i., and then only doubled over the 24-h period from 12 to 36 h p.i.

Thus, the temporal pattern of expression of the two genes differed significantly. CAT gene expression was substantial even before the onset of extensive viral DNA synthesis (6 h p.i.); β-galactosidase expression was delayed until well after the onset of extracellular virus synthesis (beginning at 10 to 12 h p.i.) and coincided with the onset of occlusion, about 20 to 24 h p.i. Relative to the levels of viral DNA in the cell (or copy number of the CAT gene), the greatest rates of CAT expression occurred between 2 and 6 h p.i. Between 6 and 18 h, the number of CAT gene copies increased over 1,000-fold, but CAT activity only doubled (see below).

AcNPV L1LC-galcat-mediated gene expression in *Drosophila* cells. Previous studies of AcNPV-mediated gene expression in the *D. melanogaster* DL-1 cell line used AcNPV L1GP-gal3 as an inoculum and monitored β-galactosidase expression from the polyhedrin promoter; no β-galactosidase was detected in these studies (28). Because the polyhedrin promoter is under strict temporal regulation, it was not clear from these studies whether the lack of β-galactosidase expression was due to the failure of AcNPV DNA to enter the *Drosophila* nucleus or the failure of AcNPV to express late occlusion-specific genes in *Drosophila* cells.

To further explore these possibilities, we infected *Drosophila* cells with AcNPV L1LC-galcat and monitored them for CAT and β-galactosidase gene expression. A mild cytopathic effect was noted in infected cells at 24 to 48 h p.i. Figure 3 shows that CAT expression was detected by 6 h p.i. and increased until 48 h p.i., after which it leveled off (see below). The maximum specific activity observed was 4.5 nmol of chloramphenicol acetylated per min per mg at 48 h p.i. This value is approximately the same as the specific activity of CAT detected in infected *S. frugiperda* cells which are fully permissive. The results show that AcNPV is capable of adsorbing, penetrating, and uncoating in *Drosophila* cells so that the viral DNA is in an expressible form. β-Galactosidase activity was not detectable in AcNPV L1LC-galcat-infected cells throughout the 48 h monitored; the assay was sensitive enough to detect 0.3% of the activity in permissive cells. This indicates that gene expression in *Drosophila* cells is promoter dependent.

AcNPV L1LC-galcat-mediated gene expression in mosquito midgut cells. Positive CAT gene expression in *Drosophila* cells suggested that AcNPV-mediated gene expression could occur in a wide range of nonpermissive insects. Since the midgut is the site of primary infection in insects, it was of practical significance to determine if midgut cells of an insect showing no substantial signs of AcNPV infection would sustain CAT gene expression on AcNPV L1LC-galcat infection of the midgut. The experiment was also of interest since many promoters, including those of retroviruses, are tissue specific in their expression.

To avoid questions concerning the amount of virus administered and its survival in food preparations or the insect

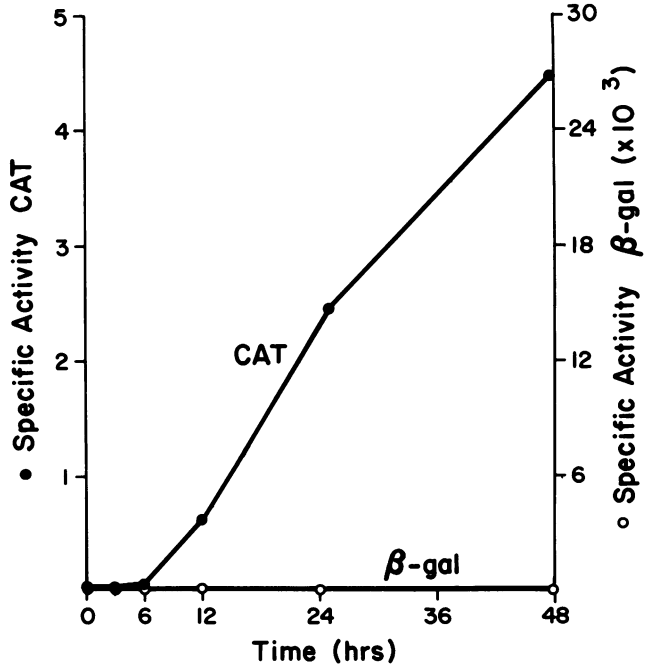

FIG. 3. Gene expression in AcNPV L1LC-galcat-infected *D. melanogaster* cells. Monolayers of *D. melanogaster* cells were infected with AcNPV L1LC-galcat at an MOI of 50. The specific activities of CAT and β-galactosidase were determined at the indicated times p.i. The points are averages of two separate but similar experiments.

foregut, the virus inoculum was introduced directly into the insect midgut. Adult female mosquitoes were inoculated with approximately 2×10^7 PFU per midgut. After 48 h infection in vivo, the midguts were removed from the mosquitoes and assayed for CAT activity and protein content. The specific activity of CAT in AcNPV L1LC-galcat-infected midgut cells at 48 h p.i. was 2.3 nmol of chloramphenicol acetylated per min per mg (Table 1). Control wild-type virus-infected midgut cells had no detectable CAT activity. These results indicate that the extracellular form of AcNPV can adsorb, penetrate, and uncoat in midgut cells of dipteran insects. Furthermore, the RSV LTR allows CAT expression in these specialized insect cells. The precise MOI was not calculated in the mosquito midgut experiment, but we estimate that it was roughly between 20 and 200 PFU per cell. The specific activity of CAT in mosquito midguts is in a similar range to that observed in *Drosophila* cell cultures (Table 1).

AcNPV L1LC-galcat-mediated gene expression in a mammalian cell culture. Observation of substantial CAT gene expression in dipteran cells raised the question of virus entry and gene expression in nontarget mammalian cells. Two previous reports had failed to detect AcNPV gene expression in mammalian cells (28, 35).

Inoculation of a mouse cell line (*M. musculus* L929) with AcNPV L1LC-galcat at an MOI of 800 failed to yield CAT gene expression. In these as well as the previous experiments in insect cells, the virus inoculum was withdrawn following the initial 1-h adsorption period. If the virus inoculum was left on the cell monolayer during subsequent incubation, a low level of CAT activity was detected. The maximum specific activity observed in mouse cell cultures was 1.5×10^{-3} nmol of chloramphenicol acetylated per min per mg at 48 h p.i. By 96 h p.i., the specific activity of CAT decreased to 70% of the 48-h expression level (data not shown). The 48-h maximum specific activity was less than 0.05% of that observed in nonpermissive dipteran cells at an equivalent MOI (Table 1). The results show that AcNPV can enter mammalian cells and express genes from a mammalian-active promoter (RSV LTR), but there appears to be a barrier at some step in the penetration or uncoating process, since the pRSVcat construct is known to be highly active in mouse cells by calcium phosphate transient expression assay (9).

Persistence of AcNPV DNA in a nonpermissive insect cell line. In an experiment designed to determine the persistence of CAT gene expression in AcNPV L1LC-galcat-infected *Drosophila* cells, infected cells were maintained for 22 days p.i., and CAT activity was assayed at various times p.i. Maximum CAT expression was observed at 48 h p.i. and continued to decrease with time; by 22 days p.i., no CAT activity could be detected (results not shown). To maintain the cells, it was necessary to dilute (1:8) the cells every 3 days. The specific activity of CAT decreased at a rate that could be accounted for by cell multiplication; e.g., the specific activity decreased to approximately one-eighth every 3 days. The initial high specific activity of CAT in infected *Drosophila* cells suggested that most cells acquired viral DNA. The decrease in CAT specific activity with time could be accounted for by the lack of viral DNA replication with cell division. More complex explanations, however, are also possible.

TABLE 1. CAT specific activities in various AcNPV L1LC-galcat-infected cells

Cell type	MOI	Specific activity of CAT[a]
S. frugiperda line	20	7.9
D. melanogaster line	50	4.5
	500	4.2
A. aegypti midgut	20–200[b]	2.3
M. musculus line	800	ND[c]
	800[d]	1.5×10^{-3}

[a] Measured at 48 h p.i.
[b] Approximate MOI 2×10^7 PFU per midgut delivered as an enema.
[c] ND, Not detectable.
[d] Virus inoculum was allowed to remain in the medium during incubation.

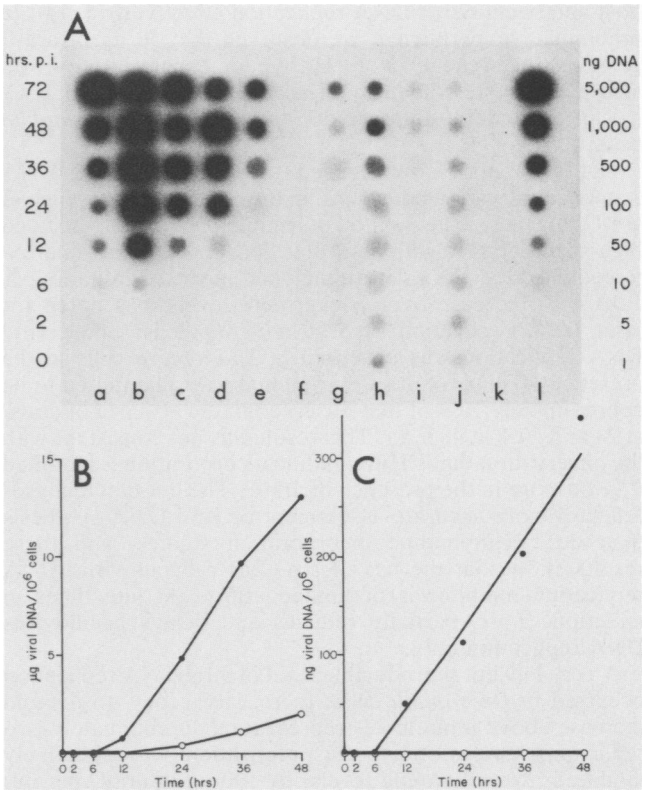

FIG. 4. The effect of ara-C on viral DNA replication in *S. frugiperda* and *D. melanogaster* cells. A dot blot (A) was prepared from total DNA extracted from *S. frugiperda* cells (lanes b through f) infected (lanes b through e) with AcNPV L1LC-galcat (MOI, 5) or mock infected (lane f) and from *D. melanogaster* cells (lanes g through k) infected with AcNPV L1LC-galcat at an MOI of 5 (lane g) or 50 (lanes h through j) or mock infected (lane k) at the times p.i. indicated to the left of the blot. Lanes c, e, and i contain 10-fold dilutions of the spots in the lanes to their left (lanes b, d, and h, respectively) to facilitate quantitation. Ara-C was present during the infections represented in lanes d, e, and j. The lanes on the far right and left (lanes a and l) are standard concentrations of AcNPV L1LC-galcat used for quantitation purposes. The blot was probed with pLC-1 labeled with ^{32}P by nick translation. The radioactive label in each spot was quantitated by scintillation counting (see the text), and the results are graphically presented in panels B (*S. frugiperda* cells) and C (*D. melanogaster* cells). Symbols: ●, Viral DNA replication in the absence of ara-C (lanes c and h); ○, viral DNA replication in the presence of ara-C (lanes e and j).

Effect of ara-C on viral DNA replication. To further understand the fate of viral DNA in *Drosophila* cells, it was of interest to determine the effects of the inhibition of DNA replication on CAT gene expression in both permissive and nonpermissive cells. Ara-C was reported by others to inhibit

host and baculovirus DNA replication effectively (5, 14). In preliminary experiments, we observed that 50 μg of ara-C per ml reduced the level of [^{3}H]thymidine incorporation into acid-precipitable counts by at least 97% in AcNPV L1LC-galcat-infected or uninfected *Spodoptera* and *Drosophila* cells.

Dot blot analysis was used to quantitate viral DNA sequences at various times p.i. in the presence or absence of ara-C (Fig. 4A). The method of sample preparation included high-speed centrifugation so that both intracellular and extracellular viral DNAs were included in the quantitation. A 1,000-fold increase over background levels was noted for viral DNA replication in *S. frugiperda* cells. Some viral DNA replication was observed in *Spodoptera* cells in the presence of 50 μg of ara-C per ml and was quantitated to be approximately 10% of that observed in the absence of ara-C at 24 and 20% at 48 h p.i. This result was not consistent with the observation that [^{3}H]thymidine incorporation is inhibited 97% or more in the presence of ara-C. The dot blot analyses reflect a more accurate assessment of viral DNA synthesis than do [^{3}H]thymidine incorporation studies, and these results show that the use of ara-C as a baculovirus DNA replication inhibitor is of limited utility. At late times in infection, ara-C partially reduces and delays baculovirus DNA replication only.

A very low but reproducible level of viral DNA replication occurred in *Drosophila* cells; there was a four- to fivefold increase above input levels representing approximately two replication cycles. Since this replication was effectively inhibited (to background levels) by 100 μg of ara-C per ml, the increase in hybridization was due to viral DNA replication. The relative increase of viral DNA above input levels in *Drosophila* cells was less than 2% of that observed in *Spodoptera* cells in the absence of ara-C (note the difference in scales in Fig. 4B and C). The total amount of viral DNA in cells infected at an MOI of 50 decreased at later times (see dot blot lane h at 72 h p.i.), suggesting some degradation of viral DNA in *Drosophila* cells after two replication cycles.

Effect of ara-C on gene expression. Although inhibition of DNA replication is not complete in AcNPV-infected *S. frugiperda* cells, the effect of ara-C inhibition on CAT and β-galactosidase gene expression in AcNPV L1LC-galcat-infected *S. frugiperda* and *D. melanogaster* cells was determined. In *Spodoptera* cells (Fig. 5, left panel), the level of CAT gene expression at 6 h p.i. was reduced 75% in the presence of ara-C, indicating an effect of DNA replication on CAT expression. With time, the observed inhibition of CAT by ara-C decreased so that by 24 h p.i. CAT gene expression reached a level almost equivalent to that observed in untreated cells. Interestingly, β-galactosidase gene expression in ara-C-treated *Spodoptera* cells was temporally regulated in an identical fashion to uninhibited cells (e.g., synthesis began at 24 h p.i.), but the specific activity was reduced 500-fold from 24 through 48 h p.i. (data not shown). Thus, the overall level of β-galactosidase (or polyhedrin) was highly dependent on the amount of viral DNA in the cell, but the level of CAT was much less sensitive to the level of DNA. The time of CAT appearance was affected by viral DNA replication, but the time of β-galactosidase appearance was not affected.

The level of CAT in AcNPV L1LC-galcat-infected *Drosophila* cells was also affected by ara-C (Fig. 5, right panel), with the greatest levels of inhibition observed at 12 h p.i. However, at later times there were approximately equal levels of CAT in both ara-C-treated and untreated AcNPV L1LC-galcat-infected *Drosophila* cells.

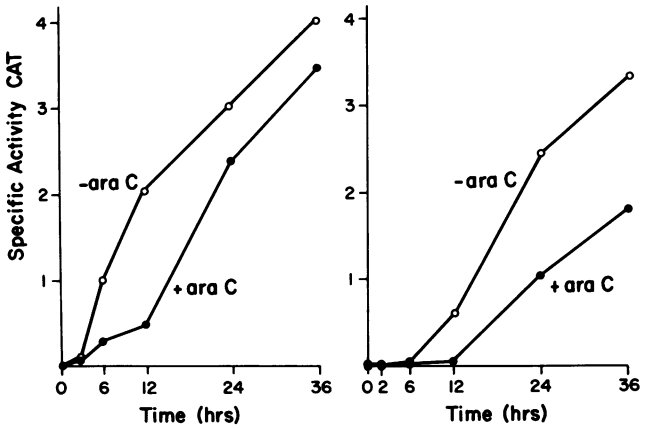

FIG. 5. Effect of ara-C on CAT gene expression in *S. frugiperda* and *D. melanogaster* cells. *S. frugiperda* cells (left panel) or *D. melanogaster* cells (right panel) were infected with AcNPV L1LC-galcat, and the infected cells were incubated in the presence (●) or absence (○) of 50 μg of ara-C per ml in the case of *S. frugiperda* and 100 μg of ara-C per ml in the case of *D. melanogaster*. The specific activities of CAT were determined at the indicated times p.i.

DISCUSSION

Although the host range of a virus is often defined in terms of the ability of the virus to replicate in a given cell, the complete replication of a baculovirus may not be as important from either an applied pesticide perspective or a safety perspective as the ability of the the virus to enter and express genetic information. For example, it has been proposed that an insect-specific neurotoxin could be recombined into a baculovirus genome, thereby making the virus more effective as a pesticide (26). The ability of the virus to enter and express the toxin in replication-refractive cells is an important consideration in expanding the host range of the virus and must also be considered in the assessment of the safety of the resulting recombinant pesticide to nontarget organisms.

In this investigation, we found that AcNPV is able to adsorb, penetrate, uncoat, and present its DNA in an expressible form to transcriptional factors of insect cells which are normally considered to be refractory to AcNPV infection. We also demonstrated that a very important component of the ability of the virus to mediate expression of its genes in nonpermissive hosts is the type of promoter controlling gene transcription.

We have confirmed the observation of Burke et al. (2) that the RSV LTR allows adjoining gene expression in *Drosophila* cells and extended the finding to mosquito and lepidopteran cells. It is probable that specific elements of this promoter are required for efficient CAT gene expression in insect cells, since Knebel et al. (17) have recently shown that the pSVOcat construct, which lacks a promoter for the CAT gene, is inefficiently expressed in *S. frugiperda* cells as determined by transient expression assays. The late polyhedrin promoter was not detectably active in nonpermissive dipteran and mammalian cells. Polyhedrin synthesis occurs very late (18 h p.i.) in the virus replication cycle and is dependent on the expression of other viral gene products (8, 15, 27) which may be dependent on interactions with host factors for activity (19, 34). We have not tested the ability of other viral promoters (7) to drive CAT gene expression in nonpermissive cells, but it is likely that the immediate early class of promoters will function in some

nonpermissive cells; a transient cytopathic effect was observed in *Drosophila* cells at 24 h p.i. with AcNPV L1LC-galcat. The nature of the promoter attached to a foreign toxin gene will clearly be a controlling factor in the ability of the recombinant virus to affect a given host.

It is particularly significant from a perspective of pesticide use that the RSV LTR allows gene expression in the midgut cells of insects, since these cells are the site of primary infection. If the effective host range of a baculovirus is to be expanded to nonpermissive hosts by introducing a toxin gene into the baculovirus genome, then it is important that the promoter controlling toxin gene expression is active in the midgut cells of the nonpermissive targets. Expression of a highly toxic protein in the midgut could also increase the efficacy of viral pesticides in permissive insects by accelerating the onset of morbidity or causing paralysis, thereby blocking feeding.

The specific activity of CAT in AcNPV L1LC-galcat-infected *Drosophila* cells was similar to that observed in permissive *Spodoptera* cells but was reduced three orders of magnitude in infected mammalian cells. Because of the possible variability in promoter strength, mRNA stability, mRNA translatability, and the stability of CAT in each cell type, it is difficult to draw definitive conclusions from the differences in specific activity. However, the RSV LTR has evolved as a powerful promoter in vertebrates and substantial expression of CAT is observed in mouse cells with the RSV LTR-CAT construct which we used (9). The RSV LTR is considered to be a relatively poor promoter in *Drosophila* sp. compared with the LTR of the transposable element *copia* (2). The most direct interpretation of our results is that much less viral DNA reaches mammalian nuclei than reaches either dipteran or lepidopteran nuclei after AcNPV L1LC-galcat infection. It is probable that there is a block in virus adsorption, penetration, or uncoating in mouse cells. The fact that the virus inoculum must be left on the cells to observe any expression suggests that the block lies at the adsorption or penetration steps.

The inability of baculovirus DNA to efficiently enter the nuclei of mammalian cells reflects an inherent level of safety of the viruses with regard to mammalian species. However, it is not advisable to introduce a gene encoding a potent mammalian toxin into baculoviruses which are to be used as pesticides. We note that a mammalian toxin gene under the control of the AcNPV polyhedrin promoter will not be expressed in mammalian cells. This aspect is relevant to the safety of baculoviruses used as vectors for foreign gene expression (25, 28, 31). Although the vector appears to be safe, we recommend that constructs be tested for expression in other mammalian cells, including human lung carcinoma cells (36), before large-scale productions are initiated. The use of CAT expression is a sensitive and direct means of addressing questions of promoter function and viral host range.

The effect of the DNA replication inhibitor ara-C on CAT gene expression in permissive and nonpermissive insect cells is interesting but not fully explicable. It appears that one or two rounds of DNA replication are necessary for timely RSV LTR-associated gene expression but that prolific viral DNA replication in permissive cells does not dramatically enhance its expression. In contrast, levels of expression from the polyhedrin promoter are dramatically influenced by ara-C inhibition of DNA replication, but the timing of expression in permissive infections is not affected. Since ara-C does not fully block viral DNA replication, more definitive conclusions about the effect of DNA replication on gene expression cannot be drawn. Previous work by other laboratories using this inhibitor should be re-evaluated, since inhibition of [^{3}H]thymidine incorporation is not a reliable means of assessing the effects of this inhibitor in baculovirus-infected cells.

The ability of baculoviruses to effectively enter and express foreign genes in distantly related insect hosts may also be relevant to the interspecies movement of transposable elements. AcNPV is known to carry transposable elements of its lepidopteran hosts (6, 23), and we have proposed that baculoviruses may play a role in interorganismal movement of these mobile elements. The viruses might reach the germ cells of refractory insects by low-level virus production (30) in the midgut or by bypassing the midgut during infection (11).

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LITERATURE CITED

1. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
2. **Burke, J. F., J. H. Sinclair, J. H. Sang, and D. Ish-Horowicz.** 1984. An assay for transient expression in transfected *Drosophila* cells using [^{3}H]guanine incorporation. EMBO J. **3**:2549–2554.
3. **Carter, J. B.** 1984. Viruses as pest-control agents, p. 375–419. *In* G. E. Russell (ed.), Biotechnology and genetic engineering reviews, vol. I. Intercept, Newcastle-upon-Tyne, United Kingdom.
4. **Casadaban, M., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *E. coli*. J. Mol. Biol. **138**:179–207.
5. **Dobos, P., and M. A. Cochran.** 1980. Protein synthesis in cells infected by *Autographa californica* nuclear polyhedrosis virus (Ac-NPV): the effect of cytosine arabinoside. Virology **103**:446–464.
6. **Fraser, M. J., G. E. Smith, and M. D. Summers.** 1983. Acquisition of host cell DNA sequences by baculoviruses: relationship between host DNA insertions and FP mutants of *Autographa californica* and *Galleria mellonella* nuclear polyhedrosis viruses. J. Virol **47**:287–300.
7. **Friesen, P. D., and L. K. Miller.** 1985. Temporal regulation of baculovirus RNA: overlapping early and late transcripts. J. Virol. **54**:392–400.
8. **Gordon, J. D., and E. B. Carstens.** 1984. Phenotypic characterization and physical mapping of a temperature-sensitive mutant of *Autographa californica* nuclear polyhedrosis virus defective in DNA synthesis. Virology **138**:69–81.
9. **Gorman, C. M., G. T. Merlino, M. C. Willingham, I. Pastam, and B. H. Howard.** 1982. The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transformation. Proc. Natl. Acad. Sci. U.S.A. **79**:6777–6781.
10. **Granados, R. R.** 1978. Replication phenomena of insect viruses *in vivo* and *in vitro*, p. 163–184. *In* H. G. Miltenburger (ed.), Safety aspects of baculoviruses as biological pesticides. Bundesministerium für Forschung und Technologie, Bonn.
11. **Granados, R. R., and K. A. Lawler.** 1981. *In vivo* pathway of *Autographa californica* baculovirus invasion and infection. Virology **108**:297–308.
12. **Gröner, A., R. R. Granados, and J. P. Burand.** 1984. Interaction of *Autographa californica* nuclear polyhedrosis virus with two nonpermissive cell lines. Intervirology **21**:203–209.

13. **Himeno, M., F. Sakai, K. Onodera, H. Nakai, T. Fukada, and Y. Kawade.** 1967. Formation of nuclear polyhedral bodies and nuclear polyhedrosis virus of silk worm in mammalian cells infected with viral DNA. Virology **33**:507–512.
14. **Kelly, D. C., and T. Lescott.** 1976. The effects of inhibitors of nucleic acid and protein synthesis on the replication of *Spodoptera frugiperda* nuclear polyhedrosis virus in cultured *Spodoptera frugiperda* cells. Intervirology **7**:360–365.
15. **Kelly, D. C., and T. Lescott.** 1981. Baculovirus replication: protein synthesis in *Spodoptera frugiperda* cells infected with *Trichoplusia ni* nuclear polyhedrosis virus. Microbiologica **4**:35–57.
16. **Klowden, M. J.** 1981. Infection of *Aedes aegypti* with *Brugia pahangi* administered by enema: results of quantitative infection and loss of infective larvae during blood feeding. Trans. R. Soc. Trop. Med. Hyg. **75**:354–358.
17. **Knebel, D., H. Lübbert, and W. Doerfler.** 1985. The promoter of the late p10 gene in the insect nuclear polyhedrosis virus *Autographa californica*: activation by viral gene products and sensitivity to DNA methylation. EMBO J. **4**:1301–1306.
18. **Lee, H. H., and L. K. Miller.** 1978. Isolation of genotypic variants of *Autographa californica* nuclear polyhedrosis virus. J. Virol. **27**:754–767.
19. **Lee, H. H., and L. K. Miller.** 1979. Isolation, complementation, and initial characterization of temperature-sensitive mutants of the baculovirus *Autographa californica* nuclear polyhedrosis virus. J. Virol. **31**:240–252.
20. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 109–112. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **McIntosh, A. H., and R. Shamy.** 1975. Effects of the nuclear polyhedrosis virus (NPV) of *Autographa californica* on a vertebrate viper cell line. Ann. N.Y. Acad. Sci. **266**:327–331.
22. **McIntosh, A. H., and R. Shamy.** 1980. Biological studies of a baculovirus in a mammalian cell line. Intervirology **13**:331–341.
23. **Miller, D. W., and L. K. Miller.** 1982. A virus mutant with an insertion of a *copia*-like transposable element. Nature (London) **299**:562–564.
24. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355, 432. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Miller, L. K.** 1981. A virus vector for genetic engineering in invertebrates, p. 203–224. *In* N. J. Panopoulos (ed.), Genetic engineering in the plant sciences. Praeger, New York.
26. **Miller, L. K., A. J. Lingg, and L. A. Bulla, Jr.** 1983. Bacterial, viral, and fugal insecticides. Science **219**:715–721.
27. **Miller, L. K., R. F. Trimarchi, D. Browne, and G. D. Pennock.** 1983. A temperature sensitive mutant of the baculovrius *Autograph californica* nuclear polyhedrosis virus defective in an early function required for further gene expression. Virology **126**:376–380.
28. **Pennock, G. D., C. Shoemarker, and L. K. Miller.** 1984. Strong and Regulated expression of *Escherichia coli* β-galactosidase in insect cells with a baculovirus vector. Mol. Cell. Biol. **4**:399–406.
29. **Sandford, K. K., W. R. Earle, and G. D. Likely.** 1948. The growth *in vitro* of simple isolated tissue cells. J. Natl. Cancer Inst. **9**:229–246.
30. **Sherman, D. E., and A. H. McIntosh.** 1979. Baculovirus replication in a mosquito (dipteran) cell line. Infect. Immun. **26**:232–234.
31. **Smith, G. E., M. D. Summers, and M. J. Fraser.** 1983. Production of human beta interferon in insect cells infected with a baculovirus expression vector. Mol. Cell, Biol. **3**:2156–2165.
32. **Summers, M., R. Engler, L. A. Falcon, and P. V. Vail (ed.).** 1975. Baculoviruses for insect pest control: safety considerations. American Society for Microbiology, Washington, D.C.
33. **Summers, M. D., and C. V. Kawanishi (ed.).** 1978. Viral pesticides: present knowledge and potential effects on public and environmental health. U.S. Environmental Protection Agency, Research Triangle Park, N.C.
34. **Summers, M. D., L. E. Volkman, and C. H. Hsieh.** 1978. Immunoperoxidase detection of baculovirus antigens in insect cells. J. Gen. Virol. **40**:545–557.
35. **Tjia, S. T., G. M. zu Altenschildesche, and W. Doerfler.** 1983. *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA does not persist in mass cultures of mammalian cells. Virology **125**:107–117.
36. **Volkman, L. E., and P. A. Goldsmith.** 1983. In vitro survey of *Autographa californica* nuclear polyhedrosis virus interaction with nontarget vertebrate host cells. Appl. Environ. Microbiol. **45**:1085–1093.