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Baculovirus Interaction with Nontarget Organisms: a Virus-Borne Reporter Gene Is Not Expressed in Two Mammalian Cell Lines

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The safety of baculoviruses with respect to mammalian species was studied by using a genetically engineered recombinant of *Autographa californica* nuclear polyhedrosis virus. This recombinant contains the chloramphenicol acetyltransferase (CAT) gene under the control of a mammalian-active promoter and expresses substantial levels of CAT activity on infection of permissive and nonpermissive insect cells (L. F. Carbonell, M. J. Klowden, and L. K. Miller, J. Virol. 56:153–160, 1985). Extremely low levels of CAT activity were detected in mouse and human cell lines that were continuously exposed to the *A. californica* nuclear polyhedrosis virus recombinant. The appearance of CAT was not inhibited by cycloheximide. Isopycnic centrifugation of purified inoculum showed that a low level of CAT activity was associated with the insect-derived viral particles. Thus, the observed CAT activity is carried into the cells with the virus inoculum, and active expression of the baculovirus-borne CAT gene is not observed in either cell line. The inability of the CAT gene to be expressed in these cell lines with this model system provides additional assurance of the safety of insect baculoviruses with respect to mammalian species.

A variety of concerns about the safety of releasing insect baculoviruses into the environment for the purpose of insect pest control have been addressed to register baculoviruses as pesticides with the U.S. Environmental Protection Agency (8, 10, 11, 26). Although several of these viruses have been approved for pesticide use by the U.S. Environmental Protection Agency (5, 16), it is of continuing interest and importance to apply the latest and most sensitive technology to the question of how baculoviruses interact with nontarget organisms (3, 27, 29). The safety issue concerning these viruses has received additional visibility recently with the release of a recombinant form of the baculovirus *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) as the first governmentally approved release of a recombinant DNA-containing organism into the environment (1). This virus is also extensively used as a vector for the expression of foreign genes (15), including oncogenes (18). Thus, the ability of Ac*M*NPV to enter and express its genetic information in nontarget organisms, including mammals, is of considerable relevance.

Electron microscopic studies have shown that Ac*M*NPV enters a variety of mammalian cells in culture (8, 9, 21, 29), but viral DNA does not persist in these cells (27), nor is there any observable effect on the chromosomal organization of mammalian cells (21). We have described the construction of a recombinant Ac*M*NPV, L1LC-galcat, which contains the *Escherichia coli* β-galactosidase gene under the control of the late Ac*M*NPV polyhedrin gene promoter and the *E. coli* chloramphenicol acetyltransferase (CAT) gene under the control of the promoter within the Rous sarcoma virus (RSV) long terminal repeat (LTR) (3). The RSV LTR-CAT gene construct that was used in those studies is expressed in a wide variety of vertebrate cells on DNA transfection (6). We first observed that the RSV LTR-CAT gene is expressed equally well in permissive lepidopteran cells and nonpermissive dipteran cells infected with L1LC-galcat, but that the polyhedrin-controlled β-galactosidase gene is expressed only in permissive lepidopteran cells. Expression is thus promoter dependent in insect cells; this conclusion was confirmed, in part, by results of direct transcriptional studies in which some early Ac*M*NPV transcripts were observed in nonpermissive dipteran cells but late Ac*M*NPV transcripts were not detected (22).

When cultured mouse cells were continuously exposed to Ac*M*NPV L1LC-galcat, very low levels of CAT activity were detected compared with the approximately 2,000-fold higher levels of CAT activity observed in permissive or nonpermissive insect cells inoculated with the same virus (3). The low levels of CAT activity were observed only after prolonged and continuous exposure to the virus. From the results of these studies we concluded that there is a block to Ac*M*NPV entry or gene expression in mouse cells; this was also confirmed in part by studies in which cell-associated CAT activity was detected in L1LC-galcat-exposed mouse cells in the presence of an inhibitor of protein synthesis (4). Volkman and Goldsmith (29) reported that a human carcinoma cell line, A549, appeared to be more susceptible to Ac*M*NPV entry than other mammalian cells, so it was of interest to extend our studies to this cell line. In this study we examined the Ac*M*NPV L1LC-galcat interaction with the human A549 cell line and also analyzed further the nature of Ac*M*NPV-mediated CAT gene expression in a mouse cell line. We show that the Ac*M*NPV-borne CAT gene is not expressed in either cell line and that the low level of CAT activity associated with these virus-exposed cells is due to presynthesized CAT associated with the virus particles in the inoculum. These data support the safety of baculoviruses with respect to mammalian species.

MATERIALS AND METHODS

Cells and media. The *Spodoptera frugiperda* IPLB-SF-21 cell line (28) was used for propagation and titering of viruses (12). The *Drosophila melanogaster* DL-1 (24) and mouse L929 (23) cell lines, as well as their growth conditions and media, have been described by Pennock et al. (20). The human lung carcinoma cell line A549 (29) was provided by Loy Volkman and was maintained at 37°C and in 5% CO_2 in Dulbecco modified Eagle essential medium (GIBCO Labo-

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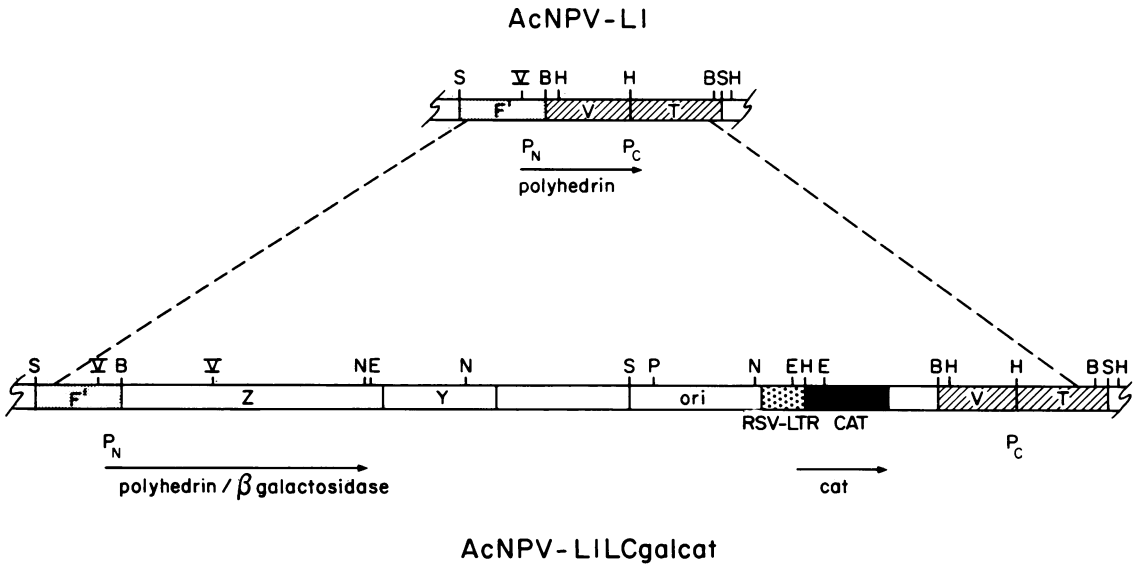

FIG. 1. Restriction map of the polyhedrin region of Ac*M*NPV L1LC-galcat. A recombinant virus, Ac*M*NPV L1LC-galcut, was constructed by allelic replacement of the Ac*M*NPV L-1 (wild-type) polyhedrin gene with sequences in plasmid pLC-1 (3). The pLC-1 plasmid contains the β-galactosidase gene fused to the polyhedrin gene and promoter; it also contains the CAT gene under control of the promoter within the LTR of RSV. L1LC-galcat carries an approximately 10-kilobase insert containing the following, from left to right: the polyhedrin promoter (F'), the polyhedrin N-terminal region (P_N) fused in-frame with the β-galactosidase gene (Z), the *lacY* gene (Y), a ColE1 origin of replication (ori), the RSV LTR promoter, the CAT gene, and a polyadenylation signal sequence from simian virus 40 followed by the C-terminal regions of polyhedrin (P_C). F', V, and T refer to the *Hin*dIII fragments of Ac*M*NPV L-1 DNA. The restriction endonuclease sites indicated are *Bam*HI (B), *Eco*RI (E), *Eco*RV (V), *Hin*dIII (H), *Nde*I (N), *Pst*I (P), and *Sal*I (S).

ratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, Utah) and antibiotics. Prior to passage and seeding, mammalian cell monolayers were dislodged by incubating in a 0.05% trypsin solution (Sigma Chemical Co., St. Louis, Mo.) containing 0.05% glucose and 4 g of NaCl, 0.2 g of KCl, and 0.29 g of $NaHCO_3$ per liter.

Viruses and inocula preparation. The L-1 variant of Ac*M*NPV served as the wild-type strain (12). The construction of a recombinant Ac*M*NPV, L1LC-galcat, has been described previously by Carbonell et al. (3), and the location of the reporter genes β-galactosidase and CAT within the polyhedrin region of L-1 is illustrated in Fig. 1. Virus used as inoculum was prepared from medium collected from infected *S. frugiperda* cells 96 h postinfection (p.i.). The virus-containing medium was centrifuged at 1,000 × *g* for 5 min to pellet cell fragments. The supernatant was further centrifuged at 86,000 × *g* for 75 min at 4°C through a cushion containing 20% sucrose in 0.1% bovine serum albumin in phosphate-buffered saline (PBSA) (12) to pellet the budded virus. The pellet was suspended in a small volume of PBSA and layered on a linear 20% to 60% sucrose density gradient also in PBSA. After centrifugation at 86,000 × *g* and 4°C for 3 h, a viral band was observed and removed from the gradient. The virus was diluted 10-fold in PBSA and repelleted through a 20% sucrose cushion in PBSA. The final pellet was suspended in the appropriate growth medium and filtered through a membrane (pore size, 0.45 μm), and the titer was determined. The *D. melanogaster* inoculum titer was 3.1×10^8 PFU/ml. The mouse cell inoculum was 4.0×10^8 PFU/ml, and the human cell inoculum was 4.2×10^8 PFU/ml.

Enzyme and protein assays. Assays for CAT activity and protein quantitation of crude cell extracts were carried out by the method of Gorman et al. (7) and Bradford (2), respectively, as modified by Carbonell et al. (3). A unit of CAT activity was defined as the amount of enzyme that acetylated 1 nmol of [^{14}C]chloramphenicol per min.

Inoculation of *Drosophila* cells. Monolayers of DL-1 cells were preincubated for 1 h with or without 100 μg of cycloheximide (Sigma) per ml and inoculated at a multiplicity of infection (MOI) of 20 PFU per cell with gentle rocking for 1 h at room temperature. The inoculum was removed and the cells were incubated at 27°C in the presence or absence of cycloheximide. Cells were collected at various times p.i.; and extracts were prepared (3), CAT activity and total protein were determined for each extract, and the specific activity of CAT was calculated.

Inoculation of mammalian cells. Monolayers of mammalian cells (10^6 cells in dishes [diameter, 35 mm]) were continuously exposed to Ac*M*NPV L1LC-galcat at an MOI of 100 PFU per cell in supplemented RPMI 1640 medium (GIBCO) (mouse cells) or supplemented DMEM medium (human cells) in the presence or absence of 100 μg of cycloheximide per ml. In some experiments, the virus inoculum (MOI, 500 PFU per cell) was removed after 1 h of incubation. Cells were collected at various times by removing the medium, washing the cell monolayer three times with PBS (12), removing the cells from the dishes with a rubber policeman, and pelleting the cells by centrifugation at 300 × *g*. Cell pellets were suspended in 100 μl of Tris hydrochloride (pH 7.8) and disrupted (3). Extracts were assayed for CAT activity and total protein, and the CAT specific activity was calculated.

Sucrose density gradient centrifugation. Sucrose gradient-purified Ac*M*NPV L1LC-galcat inoculum was layered onto another 20 to 60% linear sucrose gradient in PBS and centrifuged in an rotor (SW 28.1; Beckman Instruments, Inc., Palo Alto, Calif.) at 86,000 × *g* for 3.5 h. Twenty units of commercially available CAT (Sigma) was centrifuged in

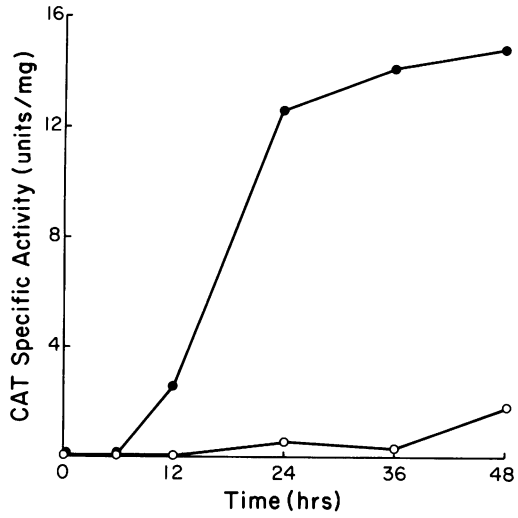

FIG. 2. CAT gene expression in *D. melanogaster* cells inoculated with Ac*M*NPV L1LC-galcat: effect of cycloheximide. *D. melanogaster* cells were inoculated with Ac*M*NPV L1LC-galcat at an MOI of 20 PFU per cell, and the inoculated cells were incubated in the presence (○) or absence (●) of 100 μg of cycloheximide per ml. CAT specific activity at various times p.i. is presented.

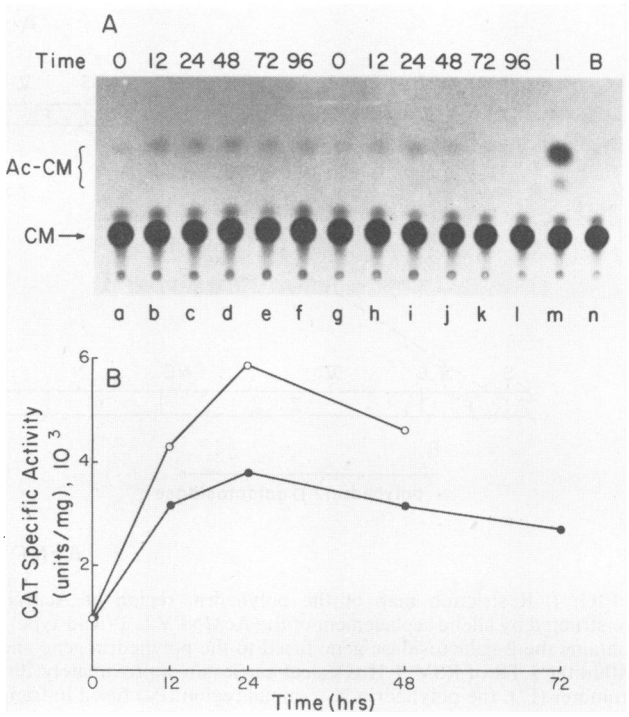

FIG. 3. Effect of cycloheximide on the appearance of CAT activity in mouse cells continuously exposed to purified Ac*M*NPV L1LC-galcat. (A) Autoradiograph of the thin-layer chromatogram resulting from ascending chromatography of [^{14}C]chloramphenicol (CM) and acetylated derivatives (Ac-CM) generated on incubation of [^{14}C]chloramphenicol, acetyl coenzyme A, and crude cell extracts of mouse cells exposed to purified Ac*M*NPV L1LC-galcat (100 PFU per cell) in the presence (lanes g through l) or absence (lanes a through f) of 100 μg of cycloheximide per ml. Cells were collected at the indicated times. Lane m resulted from assaying 2×10^7 PFU of purified inoculum (lane I), and lane n indicates a control assay without enzyme (lane B). (B) Specific activity of CAT. CAT activity was determined by quantitating the amount of acetylated [^{14}C]chloramphenicol for each lane in panel A, and the specific activity was determined by using protein quantitation data for each extract. Symbols: ○, 100 μg of cycloheximide per ml; ●, no inhibitor.

parallel. Fractions (250 μl) were collected from the bottom of the tube and freeze-thawed three times (dry ice-ethanol; 37°C). The fractions were assayed for CAT activity; this is reported as the percentage of [^{14}C]chloramphenicol acetylated by 50 μl of fraction in 1 h under standard assay conditions (see Fig. 6). The density of each fraction was calculated based on the refractive index.

RESULTS

Cycloheximide inhibits CAT gene expression in Ac*M*NPV L1LC-galcat-inoculated *D. melanogaster* cells. Ac*M*NPV L1LC-galcat contains the CAT gene under control of the promoter within the RSV LTR. Ac*M*NPV L1LC-galcat infection of nonpermissive *D. melanogaster* cells results in the production of CAT activity at levels similar to those observed in Ac*M*NPV L1LC-galcat-infected permissive lepidopteran cells (3).

If the CAT activity observed in *D. melanogaster* cells infected with L1LC-galcat is due to active CAT gene expression in these cells, then treatment of the cells with an inhibitor of protein synthesis should result in inhibition of CAT synthesis. Cycloheximide is an effective inhibitor of cytoplasmic protein biosynthesis in eucaryotic cells (19). Cycloheximide treatment effectively inhibited CAT gene expression in L1LC-galcat-infected *D. melanogaster* cells (Fig. 2). Approximately 98 to 99% inhibition of CAT synthesis was observed in the first 36 h p.i. By 48 h p.i., additional CAT activity was observed in the cycloheximide-treated cells; this was probably due to the leakiness of the inhibitor at these late times p.i.

Cycloheximide does not inhibit the appearance of CAT activity in mouse cells exposed to Ac*M*NPV L1LC-galcat. We previously observed (3) that CAT activity is inefficiently expressed in Ac*M*NPV L1LC-galcat-exposed mouse cells and is detected only if the virus inoculum is allowed to remain in contact with the cells during the exposure period.

Continuous exposure of the L929 mouse cell line to Ac*M*NPV L1LC-galcat in the absence of cycloheximide resulted in a gradual increase followed by a slight decline in CAT activity during the exposure period (Fig. 3). Even after 24 h of exposure, however, only very low levels of CAT activity were associated with the cells; the specific activity of CAT in mouse cells was approximately 0.04% of the CAT specific activity found in L1LC-galcat-inoculated *D. melanogaster* cells (notice the difference in scales between Fig. 2 and 3B).

In contrast to the pattern seen in cycloheximide-treated L1LC-galcat-inoculated *D. melanogaster* cells (Fig. 2), the appearance of CAT activity was not inhibited in L1LC-galcat-exposed L929 mouse cells treated with cycloheximide (Fig. 3). This indicates that the CAT activity observed in these cells is not dependent on protein synthesis. The protein content of the crude cell extracts steadily increased in the untreated cells (1.82 mg/ml at 48 h of exposure), whereas it rapidly decreased in the cycloheximide-treated cells (0.41 mg/ml at 48 h of exposure). CAT specific activity in cycloheximide-treated mouse cells exposed to L1LC-galcat was similar or even higher than that in the untreated cells. As early as 0 h of exposure (equivalent to the end of

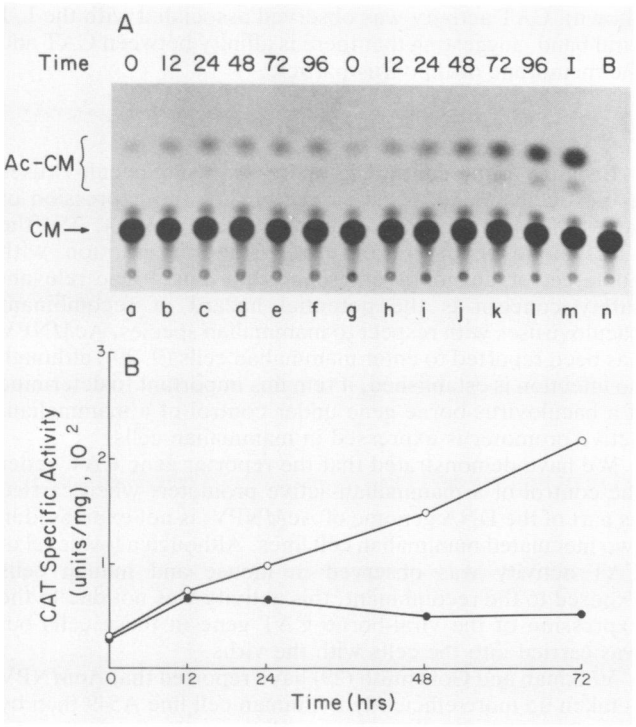

FIG. 4. Detection of CAT activity in a human lung cell line continuously exposed to purified Ac*M*NPV L1LC-galcat: effect of cycloheximide. (A) CAT assays were performed on crude cell extracts of human lung carcinoma cells exposed to Ac*M*NPV L1LC-galcat (100 PFU per cell) collected at the indicated times (lanes a through f). CAT activity detected in cells exposed to the virus in the presence of cycloheximide is shown in lanes g through l. Lane m shows activity in 50 μl (2.1×10^7 PFU) of inoculum (lane I) Lane n is a control assay without enzyme (lane B). Abbreviations: CM, Input unacetylated chloramphenicol; Ac-CM, acetylated chloramphenicol. (B) Specific activity of CAT. Symbols: ●, cells exposed to the virus in the absence of cycloheximide; ○, cells exposed to the virus in the presence of cycloheximide.

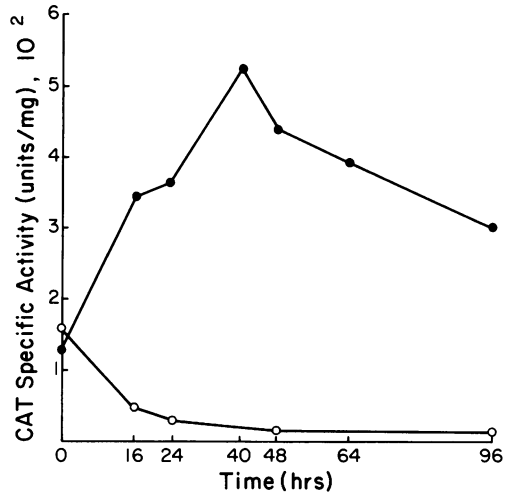

FIG. 5. Appearance of CAT activity in human cells continuously or transiently exposed to unpurified Ac*M*NPV L1LC-galcat. Monolayers of human lung cells were exposed to 500 PFU of unpurified Ac*M*NPV L1LC-galcat per cell, continuously (●) or for an initial 1-h period after which the cells were incubated in virus-free medium (○). Cells were collected at the indicated times, washed, pelleted, and disrupted. CAT specific activities were determined based on CAT activity and protein quantitations of the cell-free extracts.

the 1-h virus adsorption period), a low level of CAT activity was associated with the cells. No CAT activity was detected beyond 48 h due to loss of cell viability; the L929 mouse cells were very sensitive to cycloheximide treatment at 100 μg/ml. The lack of protein synthesis may account for the apparent increase in CAT specific activity in cycloheximide-treated cells through 48 h of exposure.

CAT gene expression is not detected in Ac*M*NPV L1LC-galcat-exposed human cells. Substantial uptake of Ac*M*NPV by the A549 human lung carcinoma cell line was demonstrated by immunological methods and was confirmed by electron microscopy, although no evidence of viral replication was obtained (29). It was of interest to determine whether the CAT gene of L1LC-galcat was expressed following uptake by A549 cells.

Cell-associated CAT activity was detected during exposure of A549 cells to high titers of purified L1LC-galcat in the absence of cycloheximide (Fig. 4A). The levels of CAT specific activity detected in human cells were only threefold higher than those observed in mouse cells (compare Fig. 4B with 3B). Cycloheximide treatment did not inhibit the appearance of CAT activity, although it did inhibit the increase in protein content of the crude cell extracts. By 72 h of exposure, the protein content in crude extracts of cyloheximide-treated cells was 0.81 mg/ml; this was less than half of that observed in the untreated cells at that time (1.90 mg/ml). Thus, the observed CAT activity was not due to the active expression of the baculovirus-borne CAT gene in the human cell line.

An additional experiment was carried out to assess the effect that time of exposure to the virus has on the levels of CAT activity observed in L1LC-galcat-exposed A549 cells. The virus inoculum used in this case was not purified by banding on a sucrose gradient as was done for all other experiments. Continuous exposure of human lung (A549) cells to high titers (MOI, 500 PFU per cell) of unpurified L1LC-galcat resulted in an increase in CAT specific activity which was maximal at 40 h of exposure and gradually declined through 96 h of exposure (Fig. 5). When the inoculum was removed after 1 h of exposure (0 h), CAT activity was observed and subsequently decreased so that by 24 h of exposure only 15% of the specific activity observed at 0 h remained. This indicates that the observed increase in CAT specific activity with time reflects a slow adsorption and subsequent accumulation of preexisting CAT in A549 cells which are continuously exposed to L1LC-galcat. These data thus corroborate the results of the cycloheximide studies.

Detection of CAT activity in sucrose gradient-purified Ac*M*NPV L1LC-galcat inocula. The observation that a low level of CAT activity is detected in both mouse and human cells immediately after exposure to L1LC-galcat and that cycloheximide is not inhibitory to expression of CAT in these cells suggests that the observed CAT activity is associated with the virus inocula. The inocula used in these experiments were purified extensively by banding on a sucrose gradient, repelleting, and resuspending in the appropriate medium to eliminate the CAT activity which might be present in the medium of the L1LC-galcat-infected cells. Nevertheless, CAT activity was observed in the virus inocula for the mouse cell inoculum (Fig. 3A, lane m) and for the human cell inoculum (Fig. 4A, lane m). The amount of

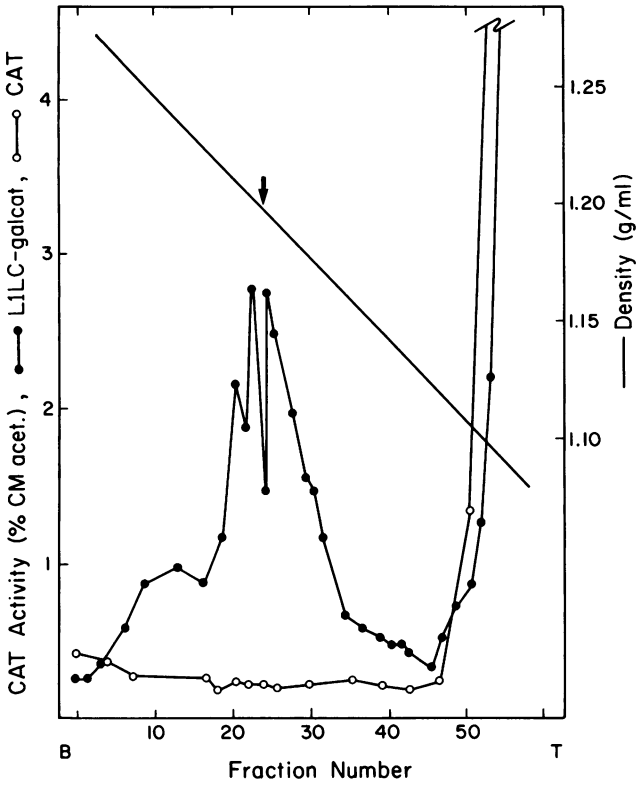

FIG. 6. CAT activity profiles of Ac*M*NPV L1LC-galcat or commercial CAT centrifuged on a sucrose density gradient. Twenty units of commercially available CAT (○) and 10^9 PFU of sucrose gradient-purified Ac*M*NPV L1LC-galcat (●) were centrifuged through sucrose density gradient. Fractions were collected and assayed for CAT activity, which is reported here as the percentage of [^{14}C]chloramphenicol acetylated (% CM acet.). The density of each fraction was determined by refractometry. The arrow points to the density at which the budded virus was observed. Abbreviations: B, Bottom; T, top.

virus assayed for both mouse and human cell inocula was estimated to be approximately 2.0×10^7 PFU (see above) and yielded an average of 1.4×10^{-2} U of CAT activity per 10^8 PFU.

CAT activity associated with Ac*M*NPV L1LC-galcat viral particles. To ascertain that the CAT activity observed in the L1LC-galcat inocula was associated with the viral particles, sucrose gradient-purified L1LC-galcat was banded a second time on a sucrose density gradient, and fractions were collected and assayed for CAT activity. CAT activity was associated with the viral band resulting from isopycnic centrifugation of purified L1LC-galcat (Fig. 6). The viral band was identified visually and by A_{260} measurements of the collected fractions. The viral particles had a buoyant density of approximately 1.99 g/ml (circa 44.5% sucrose), which agrees closely with a previously reported density (12). In a parallel gradient, commercially available CAT remained at the top of the gradient (Fig. 6). The CAT activity observed at the top of the gradient in the L1LC-galcat gradient may have been due to free CAT released from partially disrupted viral particles or may indicate that the CAT activity was only weakly associated with the viral particles.

The location of CAT in the viral particles was further investigated by isopycnic centrifugation of wild-type (L-1) inoculum mixed with 20 U of commercial CAT (data not shown). CAT activity was observed associated with the L-1 viral band, suggesting that there is affinity between CAT and the membrane of the virus particle.

DISCUSSION

Because of the continuing interest in using baculoviruses as pesticides (16, 17) and as vectors for the expression of genes of commercial and scientific value (13, 14, 25), the assessment of their safety regarding interaction with nontarget organisms is of prime importance. One relevant safety concern is the potential hazard of recombinant baculoviruses with respect to mammalian species. Ac*M*NPV has been reported to enter mammalian cells (9, 29); although no infection is established, it remains important to determine if a baculovirus-borne gene under control of a mammalian-active promoter is expressed in mammalian cells.

We have demonstrated that the reporter gene CAT under the control of a mammalian-active promoter, when carried as part of the DNA genome of Ac*M*NPV, is not expressed in two inoculated mammalian cell lines. Although a low level of CAT activity was observed in mouse and human cells exposed to the recombinant, this activity was not due to the expression of the viral-borne CAT gene in these cells but was carried into the cells with the virus.

Volkman and Goldsmith (29) have reported that Ac*M*NPV is taken up more efficiently by human cell line A549 than by any of several other vertebrate cell lines. We found that the levels of CAT specific activity detected in human A549 cells were two- to threefold higher than those observed in mouse L929 cells when exposed to L1LC-galcat. This may reflect the slightly higher efficiency of virus uptake in A549 cells. However, Volkman and Goldsmith (29) also have reported that it is the polyhedra-derived virus form of Ac*M*NPV that is taken up more efficiently by human A549 cells than the budded virus form. We have used the budded virus form in our studies because Ac*M*NPV L1LC-galcat does not form polyhedral occlusion bodies. Thus, results of our study have not eliminated the possibility that a polyhedra-derived virus form of L1LC-galcat or another polyhedrin-substitution Ac*M*NPV recombinant may be more efficient in entering A549 cells. This may be of minimal concern because the polyhedra-derived virus form only exists transiently in nature in the alkaline environment of infected larval midguts, and it is therefore unlikely that mammalian cells would come in contact with this form of the virus.

Results of this study corroborate those of previous reports that affirm the safety of baculoviruses with respect to vertebrates, especially mammals, and show that although Ac*M*NPV may gain entry into the cell, the viral DNA either does not reach the nucleus or is not available to cell transcriptional factors. The RSV LTR-CAT construct used in our experiments is actively expressed in a variety of mammalian cells, including mouse NIH 3T3 and HeLa cells (6), when delivered as plasmid DNA via calcium phosphate-mediated transfection. When it gains access to the cell as part of the Ac*M*NPV genome, however, the RSV LTR-CAT construct is not expressed. All accumulated evidence suggests that insect baculoviruses pose no threat to nontarget organisms.

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