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# Structural and Intracellular Proteins of the Nonoccluded Baculovirus HZ-1

JOHN P. BURAND,* BRAD STILES, AND H. ALAN WOOD
*Boyce Thompson Institute for Plant Research, Ithaca, New York 14853*

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A plaque-purified isolate of the baculovirus HZ-1 was used to examine the kinetics of replication of this persistent, nonoccluded virus in TN-368 cells. Twenty-eight virus structural proteins ranging in molecular weight from 153,000 to 14,000 were identified. Fourteen of these proteins were found to be glycosylated. The sequence of appearance of the 37 virus-induced intracellular polypeptides was determined by pulse-labeling with [$^{35}$S]methionine. *N*-[$^{3}$H]acetylglucosamine, [$^{3}$H]mannose, and the glycosylation inhibitor tunicamycin were used to detect virus structural glycoproteins. Post-transcriptional modification of two virus-induced proteins was detected.

The nonoccluded baculovirus HZ-1 was originally isolated as a persistent virus from the lepidopteran cell line IMC-HZ-1 (5). This virus is also capable of persisting in a continuous cell line of *Trichoplusia ni* cells (TN-368) (1a, 12). Like the nuclear polyhedrosis viruses (NPVs), HZ-1 virus contains a circular, double-stranded DNA genome and replicates in the nucleus of susceptible cells (1, 7, 13). However, unlike the nonoccluded form of NPVs, which is released from virus-infected cells by budding, HZ-1 virus is released by lysis of the nuclear and cytoplasmic membranes (12; R. R. Granados, manuscript in preparation). The genome of HZ-1 virus is approximately 154 × $10^6$ daltons (1a, 7), which is about twice the size reported for other baculoviruses (13).

In animal virus systems, the study of virus-induced intracellular polypeptides has permitted analysis and understanding of the molecular events involved in virus replication. Until now the identification and characterization of the virus-specific intracellular polypeptides of baculoviruses have been limited to the two closely related viruses *Autographa californica* MNPV (3, 4, 11, 15) and *T. ni* MNPV (8). In this study we examined the synthesis of HZ-1 virus-specific intracellular and structural proteins by following the incorporation of [$^{35}$S]methionine in infected cells. The incorporation of [$^{3}$H]mannose and *N*-[$^{3}$H]acetylglucosamine was used to identify glycosylated structural proteins.

## MATERIALS AND METHODS

**Cells and virus.** *T. ni* (TN-368) tissue culture cells were maintained at 26°C in a modified TMNFH medium (16), in which 2.6 g of tryptone (Difco Laboratories) per liter was substituted for the lactalbumin hydrolysate and yeastolate of TMNFH (6). A plaque-purified virus isolate designated HZ-1-B1 (1a) recovered from a TN-368 cell line persistently infected with IMC-HZ-1 virus was used in this study. This virus was propagated in TN-368 cells as outlined by Burand et al. (1a).

**Virus growth curve.** Virus inoculation was carried out after the centrifugation-inoculation procedure outlined by Wood (14). Multiwell plates (Falcon Plastics, Oxnard, Calif.), containing 3 × $10^5$ log-phase cells per well, and virus at a multiplicity of infection (MOI) of 20 PFU/cell were centrifuged for 1 h at 1,000 × *g*. After centrifugation, the cells were washed with medium, and the plates were incubated at 26°C. Immediately after these washes (zero time) and at various times post-inoculation (p.i.), the contents of the well were fractionated into two samples. The culture supernatant was collected and designated the extracellular virus (ECV) sample. The cells remaining in the well were resuspended in 1.0 ml of medium and sonicated at 50 W for 10 s in a bath sonicator (model W185 cell disrupter; Heat Systems, Plainview, N.Y.); these were designated cell-associated virus (CAV) samples. All samples were stored on ice until plaque assayed.

**Plaque assay.** Titers of both ECV and CAV samples were determined by a slight modification of the plaque assay procedure outlined by Wood (14). The cell concentration used for the plaque assay of HZ-1-B1 virus was 4 × $10^5$ cells per well, and after centrifugation cells were overlaid with 2.0% Sea-plaque agarose (Marine Colloids, Rockland, Maine). After 3 days of incubation at 26°C, 0.1 ml of trypan blue dye (5%, wt/vol) was added to each well. The excess dye was removed after 5 min, and the plates were incubated at 26°C for an additional 24 h before the plaques were counted.

**[$^{35}$S]methionine labeling of intracellular polypeptides.** The synthesis of intracellular polypeptides after infection with HZ-1 was determined through the incorporation of [$^{35}$S]methionine as outlined by Wood (15). Virus- and mock-infected cells, inoculated as outlined

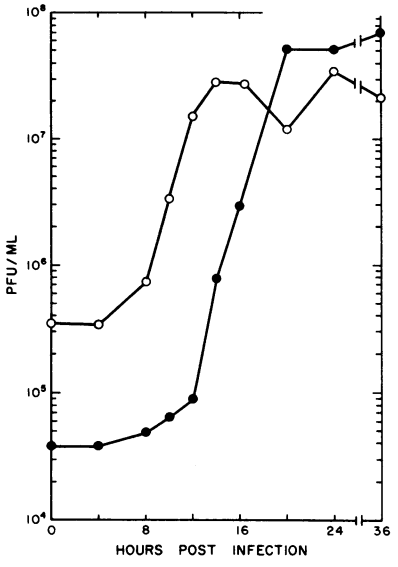


FIG. 1. Growth curve of HZ-1-B1 virus in TN-368 cells. Cells were infected at an MOI of 20 PFU/cell, and the levels of infectious CAV (○) and ECV (●) from 0 to 36 h p.i. were measured by plaque assay.

above, were first starved in Grace medium without amino acids for 1 h and then labeled for 1 h with 5 μCi of [$^{35}$S]methionine (12.5 μCi/ml) in the same medium. After being labeled, the cells were harvested and prepared for polyacrylamide gel electrophoresis (PAGE).

**Detection of post-translational modification.** In pulse-chase experiments, cells were starved for 1 h as described, pulse-labeled with [$^{35}$S]methionine for 1 h at 4, 5, or 6 h p.i., and then chased with TMNFH medium for 15 or 30 min or for 1, 2, 6, or 12 h before being harvested.

**Labeling of virus structural proteins.** HZ-1 virus-infected cells were prepared as for the virus growth curve study. At 8 h p.i., 10 μCi of [$^{35}$S]methionine (10 μCi/ml) was added to each well. The ECV was collected at 24 h p.i. from the infected cell culture supernatant by centrifugation at 15,600 × *g* in an Eppendorf microfuge for 30 min and then processed for PAGE.

**Structural glycoproteins.** TN-368 cells (2 × $10^7$ per 150-cm$^2$ flask) were allowed to attach and then inoculated with HZ-1 virus at an MOI of 2. The flasks were rocked slowly for 1 h, and additional medium or medium plus tunicamycin (TM) (final concentration, 10 μg/ml) was added. At 8 h p.i. the medium was removed, and 10 ml of low-sugar medium (with and without 10 μg of TM per ml) was added. Low-sugar medium consisted of Grace solution prepared with only 1/10 the normal glucose content, no fucose or sucrose, and 10% dialyzed fetal calf serum (osmotic concentration adjusted with sorbitol). Either D-[2-$^3$H]mannose (10 to 20 Ci/mmol; New England Nuclear Corp.) or *N*-acetyl-D-[1-$^3$H]glucosamine (2 to 10 Ci/mmol; Amersham Corp.) was added to give a final concentration of 40 μCi/ml in each flask. At 24 h p.i., 10 ml of complete medium (with and without TM) was added, and the cells were incubated for an additional 24 h.

Medium containing labeled virus was collected and centrifuged at 800 × *g* for 5 min to remove cells. The virus was pelleted through a 5-ml underlay of 20% (wt/wt) sucrose in 0.001 M Tris-hydrochloride (pH 7.5) by centrifugation at 66,000 × *g* for 1 h at 5°C. The pellet was suspended in the Tris buffer, layered over 25 to 55% (wt/wt) sucrose (in the Tris buffer) density gradients, and centrifuged at 150,000 × *g* for 4 h at 5°C in an SW41 rotor. The virus band was collected, diluted fourfold with the Tris buffer, and pelleted by centrifugation at 76,000 × *g* for 1 h at 5°C. Virus was suspended in 50 μl of the Tris buffer. Samples containing equivalent units of TM-treated and untreated virus as measured by optical density at 260 nm were subjected to PAGE.

**PAGE and autoradiography.** Cells and ECV for PAGE were disrupted in dissociation buffer by being heated to 100°C for 3 min as described by Laemmli (9). Samples containing $10^5$ cpm of incorporated [$^{35}$S]methionine were electrophoresed in 11% polyacrylamide vertical slab gels (15 by 16 by 0.15 cm) in the presence of 0.1% sodium dodecyl sulfate by the procedure of Laemmli (9) at 6 W per gel (15). After electrophoresis, gels were dried under vacuum, and labeled proteins were detected by autoradiography with Kodak X-Omat RP X-ray film. For $^3$H-labeled glycoproteins, gels were pretreated with En$^3$Hance (New England Nuclear) before being dried, and the X-ray film was preexposed as described by Laskey (10) before autoradiography. Autoradiograms were analyzed with a Quick Scan R&D densitometer (Helena Laboratories, Beaumont, Tex.), and the approximate molecular weights of the proteins were determined from gel scans, with $^{14}$C-methylated phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrose, and lysozyme (Amersham) used as molecular weight standards.

## RESULTS

**Virus replication in cell culture.** The replication cycle of HZ-1 virus in TN-368 cells is shown in Fig. 1. Infectious CAV was first detected at about 8 h p.i. At this time, virus-infected cells appeared rounded, with enlarged nuclei (data not shown). Infectious ECV was first detected between 10 and 12 h p.i. During this time approximately 20 to 30% of the cells were lysed. The maximal CAV titer occurred between 14 and 16 h, whereas ECV reached a peak titer at about 20 h p.i. By 24 h p.i. between 80 and 90% of the cells were lysed.

**Synthesis of virus-induced intracellular polypeptides.** HZ-1 virus-specific intracellular proteins synthesized at various times p.i. were studied by following [$^{35}$S]methionine incorporation. The labeled proteins in mock-infected and virus-infected TN-368 cells are shown in Fig. 2. There were 37 virus-induced polypeptides detected in the first 12 h p.i. (Table 1).

At 2 h p.i., a 75,000-dalton (75K) virus-specific protein was detected. This protein was not

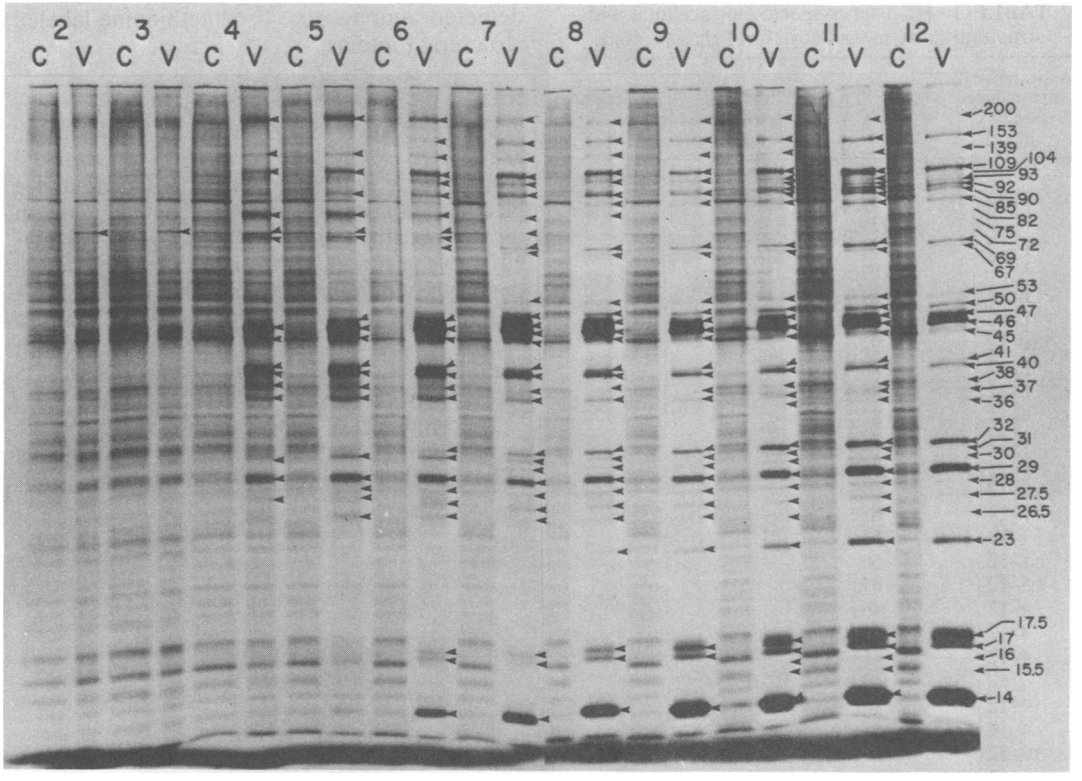


FIG. 2. Autoradiogram of intracellular polypeptides of control (C) and HZ-1 virus (V) pulse-labeled for 1 h with [$^{35}$S]methionine. TN-368 cells were infected at an MOI of 20 PFU/cell and starved for 1 h before the addition of label. After being labeled for 1 h during the indicated time p.i., samples were collected and analyzed by PAGE. The apparent molecular weights ($10^3$) of the virus-specific intracellular polypeptides are shown at the right.

detected after 7 h p.i. In addition, 25 other proteins were identified before 7 h p.i., before infectious CAV was produced or ECV was released from the cells. Most of the proteins initially made during this time were made throughout virus infection. However, the 72K, 75K, and 82K proteins were not detected after 6, 7, and 8 h p.i., respectively, and synthesis of the 104K proteins was terminated by 12 h p.i. After CAV and ECV titers began to increase (8 h p.i.), an additional 11 virus-specific proteins were detected. All of these proteins were synthesized throughout the remainder of the virus replication cycle. The level of [$^{35}$S]methionine incorporation decreased significantly after 14 h p.i. (data not shown), indicating a significant decrease of protein synthesis in virus-infected cells.

**Post-transcriptional modification.** Pulse-chase experiments in which virus-infectd cells were pulse-labeled with [$^{35}$S]methionine at either 4, 5, or 6 h p.i. and then chased in medium containing methionine for 15, 30, 60, 90, or 120 min showed no evidence of post-transcriptional modification of the intracellular virus proteins. However. when a 1-h label at 4 h p.i. was followed by 6- or 12-h chase periods, the label in the 109K and 82K proteins appeared to decrease. During these chase periods, there was an increase in the amount of label detected in a protein band of approximately 107K (Fig. 3).

**Virus structural proteins.** HZ-1 virus structural proteins were detected by [$^{35}$S]methionine labeling of virus-infected cells between 8 and 24 h p.i. Labeled ECV was recovered from infected cell culture supernatants and compared with virus-specific intracellular polypeptides (Fig. 4, lane B; Table 1). Twenty-six virus structural proteins were detected by this method. The proteins that were not visible in the photographic reproductions were consistently detected by autoradiography. Therefore, these proteins were considered to contain low levels of methionine or to be minor structural proteins, or both. All of these structural proteins, except the 107K protein, corresponded with virus-specific intracellular polypeptides synthesized during the 12th h p.i.

**Structural glycoproteins.** A total of 14 HZ-1

TABLE 1. HZ-1 virus-specific intracellular and structural proteins and structural glycoproteins

| Appearance of virus-induced proteins (h p.i.) | Mol wt ($10^3$) | Structural protein | Structural glycoprotein |
|---|---|---|---|
| 4–12 | 200 | | |
| 6–12 | 153 | + | + |
| 4–12 | 139 | | |
| 4–12 | 109 | + | + |
| | 107 | + | |
| 6–11 | 104 | | |
| 10–12 | 93 | + | |
| 10–12 | 92 | + | |
| 5–12 | 90 | | |
| 8–12 | 85 | + | |
| 4–8 | 82 | | |
| 2–7 | 75 | | + |
| 4–7 | 72 | | + |
| 6–12 | 69 | + | + |
| 7–12 | 67 | + | + |
| 7–12 | 53 | + | |
| 7–12 | 50 | + | |
| 5–12 | 47 | | |
| 4–12 | 46 | + | + |
| 4–12 | 45 | + | + |
| 4–12 | 41 | | |
| 4–12 | 40 | + | + |
| 4–12 | 38 | | |
| 4–12 | 37 | + | |
| 10–12 | 36 | + | |
| 6–12 | 32 | + | |
| 4–12 | 31 | + | |
| 7–12 | 30 | | |
| 4–12 | 29 | + | |
| 5–12 | 28 | + | |
| 4–12 | 27.5 | + | + |
| 5–12 | 26.5 | | |
| 8–12 | 23 | + | + |
| 6–12 | 17.5 | + | |
| 6–12 | 17 | + | |
| 10–12 | 16 | + | + |
| 10–12 | 15.5 | + | + |
| 6–12 | 14 | + | + |

structural proteins were found to be glycosylated after being labeled with both [$^3$H]mannose and *N*-[$^3$H]acetylglucosamine (Fig. 4, lanes C and E; Table 1). Several of the *N*-acetylglucosamine bands were quite faint (Fig. 4, lane E). All major structural proteins were identified after analysis of [$^{35}$S]methionine-labeled virus produced in the presence of TM (data not shown). Nevertheless, TM-grown virus lacked significant incorporation of *N*-[$^3$H]acetylglucosamine (Fig. 4, lane D), confirming that these structural glycoproteins were glycosylated via *N*-glycosidic bonds. Twelve of the glycoproteins corresponded with HZ-1 structural proteins identified by [$^{35}$S]methionine incorporation. However, the 75K and 72K structural glycoproteins were not detected among the [$^{35}$S]methionine-labeled structural proteins.

## DISCUSSION

The replication of HZ-1 virus in TN-368 cells, as reported by Granados et al. (5), was examined more closely with a cloned virus isolate and a plaque assay system. A significant increase in the level of PFU with CAV samples was detected between 8 and 10 h p.i. (Fig. 1), indicating that virus maturation occurs within the cell 3 to 4 h before the level of infectious virus begins to increase in ECV samples. This is consistent with the observation that infectious HZ-1 virus is released by cell lysis (5).

The sequence of appearance of virus-specific intracellular proteins was examined by labeling HZ-1-infected cells with [$^{35}$S]methionine. Ami-

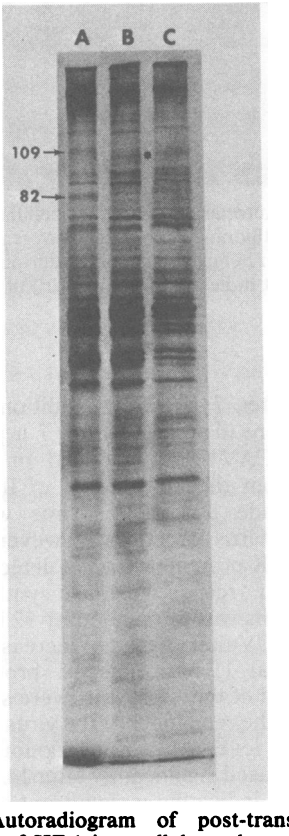


FIG. 3. Autoradiogram of post-transcriptional modification of HZ-1 intracellular polypeptides. TN-368 cells infected at an MOI of 20 PFU/cell were pulse-labeled for 1 h at 4 h p.i. with [$^{35}$S]methionine and then chased with complete medium for (A) 0, (B) 6, or (C) 12 h before being analyzed by PAGE. Arrows, Virus-specific proteins which appear to be modified, with apparent molecular weights ($10^3$). *, 107 K protein, a possible post-transcriptional modification product.

no acids labeled with $^{14}C$ gave essentially the same results as obtained with [$^{35}S$]methionine label; however, the activity obtained with these compounds was much lower than that obtained with the $^{35}S$-labeled amino acid. Based on the appearance of these proteins, the replication cycle of the virus was divided into three stages: early (0 to 4 h p.i.), intermediate (4 to 8 h p.i.), and late (8 to 13 h p.i.). During the early stage of virus replication, only the 75K protein was detected. The majority (29 proteins) of the virus-induced intracellular proteins were first detected during the intermediate stage of HZ-1 replication. Two types of viral control over protein synthesis can be seen during the intermediate stage. The first is a regulation of host cell protein synthesis. By about 7 h p.i., host cell proteins were not detected in virus-infected samples. This suggests that HZ-1 may be capable of shutting off host cell protein synthesis early in the virus infection cycle. The second type of regulation observed is a temporal control of virus-specific intracellular proteins. During this intermediate phase of viral replication, a sequential turning on and turning off of three virus-specific proteins (72K, 75K, and 82K) was detected. The late stage of virus replication is characterized by maturation of the virus, culminating in cell lysis with the release of infectious virus. Seven virus-specific proteins were first detected during this stage. All of these proteins were present during the entire late stage and were identified as virus structural proteins.

Post-translational modification of two HZ-1 virus-specific intracellular polypeptides (109K and 82K) was detected. However, these changes could only be detected when long chases (6 or 12 h) were used. The 109K protein is a minor structural protein, which is highly glycosylated as an intracellular protein (Stiles et al., unpublished data). Since the 107K protein is a major structural protein (Fig. 4, lane B), is not heavily glycosylated (Fig. 4, lane E), and is only observed during virus-induced protein synthesis studies after long chase periods, we suspect that the 107K protein may arise through deglycosylation of the 109K protein. The detection of post-translational modification only late in virus infection suggests its possible involvement in virus maturation. However, further experiments are being conducted to determine the exact nature of these modifications and what role, if any, post-transcriptional modification has in the formation of mature infectious HZ-1 virus.

Since HZ-1 virus is released from the cell by lysis, it was not possible to obtain virus preparations free of contaminating host cell proteins. For this reason we identified the structural proteins of this virus by radiolabeling infected cells at 8 h p.i. and examining the labeled proteins in

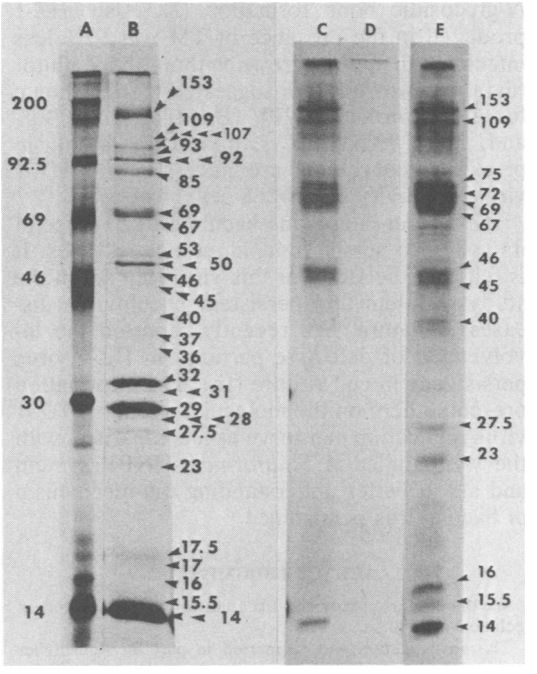


FIG. 4. HZ-1 virus structural proteins and glycoproteins. (A) Molecular weight standards; (B) [$^{35}S$]methionine-labeled viral proteins; (C) *N*-[$^{3}H$]acetylglucosamine- and (E) [$^{3}H$]mannose-labeled viral glycoproteins analyzed by PAGE. Lane D, HZ-1 virus labeled with *N*-[$^{3}H$]acetylglucosamine in the presence of 10 μg of TM per ml. In this lane, the virus particle concentration was approximately equal to that in lane C. Arrows, Virus proteins and their apparent molecular weights ($10^3$).

ECV harvested at 24 or 48 h p.i. By the addition of labeled amino acids or sugars to infected cells after host cell protein synthesis was turned off, only virus-specific proteins were detected in autoradiograms of ECV preparations. We identified 28 HZ-1 virus structural proteins in this manner. Of these, 26 were labeled with [$^{35}S$]methionine and 14 with $^{3}H$-labeled sugars. Only two structural glycoproteins (72K and 75K) did not have [$^{35}S$]methionine-labeled structural protein counterparts. These two structural glycoproteins corresponded to the virus-specific intracellular proteins detected by [$^{35}S$]methionine labeling early in the replication of the virus. The 28 proteins identified here represent a minimum number of HZ-1 virus structural proteins.

The glycosylation inhibitor TM has proven to be a useful tool in the study of HZ-1 virus glycoproteins. Virus grown in the presence of TM and $^{3}H$-labeled sugars did not show any radioactivity incorporated into viral protein. This showed that the labeled sugars were not significantly metabolized by the cells and that glycosylation of the viral proteins occurred via

*N*-glycosidic bond formation (2). Also, HZ-1 produced in the presence of TM was 97% less infectious than virus grown without the inhibitor (data not shown). This suggested that as shown for *A. californica* *M*NPV (B. Stiles, J. P. Burand, and H. A. Wood, J. Invertebr. Pathol., in press), glycoproteins are necessary for HZ-1 virus infectivity in TN-368 cells.

HZ-1 is an interesting baculovirus because of its ability to persist in lepidopteran cell lines. It is felt that the study of this virus will aid in the study of latent and persistent baculovirus diseases in nature. We recently reported the involvement of defective particles in HZ-1 virus persistence in cell culture (1a). The information presented here on the molecular events of HZ-1 virus replication can serve as a comparison with the well-studied *A. californica* *M*NPV system and aid in better understanding the mechanism of baculovirus persistence.

## ACKNOWLEDGMENTS

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