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Synthesis of Biologically Active Influenza Virus Hemagglutinin in Insect Larvae

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The hemagglutinin of influenza (fowl plague) virus was expressed in larvae of *Heliothis virescens* by using recombinant *Autographa californica* nuclear polyhedrosis virus (AcNPV) as a vector. Animals were infected with the recombinant virus either by parenteral injection or by feeding. For oral uptake, recombinant virus occluded in polyhedra obtained from cultured *Spodoptera frugiperda* cells after coinfection with authentic AcNPV was used. Immunohistological analyses of infected animals revealed that the hemagglutinin was expressed only in those tissues that are also permissive for the replication of authentic AcNPV. These tissues included hypodermis, fat body, and tracheal matrix. After oral infection, hemagglutinin was also detected in individual gut cells. The amount of hemagglutinin synthesized in larvae after parenteral infection was 0.3% of the total protein, compared with 5% obtained in cultured insect cells. The hemagglutinin was transported to the cell surface and expressed in polarized cells only at the apical plasma membrane. It was processed by posttranslational proteolysis into the cleavage products HA_1 and HA_2. Oligosaccharides were attached by *N*-glycosidic linkages and were smaller than those found on hemagglutinin obtained from vertebrate cells. Hemagglutinin from larvae expressed receptor binding and cell fusion activities, but quantitation of the hemolytic capacity revealed that it was only about half as active as hemagglutinin from vertebrate or insect cell cultures. Chickens immunized with larval tissues containing hemagglutinin were protected from infection with fowl plague virus. These observations demonstrate that live insects are able to produce a recombinant membrane protein of vertebrate origin in biologically active form.

Baculovirus vectors that have been developed mainly on the basis of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) have found wide application for the expression of foreign genes in insect cells. Under the control of the potent polyhedrin promoter, gene expression is particularly efficient, with protein yields often significantly higher than in bacterial, yeast, or vertebrate expression systems (9). Furthermore, the available evidence indicates that insect cells are able to process recombinant proteins similarly to higher eucaryotic cells. For instance, we and others have recently demonstrated that the hemagglutinin of influenza virus, which is an integral membrane protein, undergoes glycosylation, proteolytic cleavage, and directed transport to the cell surface when expressed by a baculovirus vector and that it displays receptor-binding, membrane fusion, and antigenic properties like the authentic protein (8, 14; K. Kuroda, R. Rott, W. Doerfler, and H.-D. Klenk, *in* I. Mitsuhashi, ed., *Invertebrate Cell System Applications*, in press). Similarly, a whole series of secretory proteins, such as human beta interferon (18) and human interleukin-2 (19), have been obtained in biologically active form in this system. The baculovirus vector also compares favorably with all vertebrate expression systems in that it is not pathogenic for mammals and does not employ transformed cells. For these reasons, the system promises to be suitable for vaccine production, and indeed, human immunodeficiency virus glycoprotein derived from recombinant baculovirus has provided the first experimental acquired immunodeficiency syndrome vaccine to be used in human trials (6, 9).

In our previous study, influenza virus (fowl plague virus [FPV]) hemagglutinin was obtained from cultured *Spodoptera frugiperda* cells infected with recombinant AcNPV (8). We have now used the same vector to express the hemagglutinin in whole larvae of *Heliothis virescens* (Lepidoptera, Noctuidae). By a similar approach, production of several secretory proteins has been accomplished in silkworms (5, 10, 11, 13). We will demonstrate that living *Heliothis virescens* larvae can be infected either parenterally with free virus particles or orally with virions occluded in polyhedra. Hemagglutinin was synthesized in biologically active form, and the larval tissues suitable for production have been identified.

MATERIALS AND METHODS

Cell cultures and viruses. The insect cell line from *Spodoptera frugiperda* was propagated in TC-100 medium which was modified as previously reported (2) and contained 10% fetal calf serum. The methods for the propagation of AcNPV (2, 20) and the recombinant AcNPV-HA (8) cell cultures have been described before. FPV strain A/FPV/Rostock/34 (H7N1) was grown in MDCK cells (16). Seed stocks were obtained from the allantoic cavities of 11-day-old embryonated eggs (7).

Isolation of polyhedra from infected *S. frugiperda* cell culture. Three days after infection, polyhedra were purified from cell cultures that had been inoculated with authentic AcNPV alone or with both authentic and recombinant AcNPV by pelleting at 10,000 × *g* for 20 min, incubation at 23°C for 30 min in a 0.5% sodium dodecyl sulfate (SDS) solution, and then by a series of washes in water, in 0.5 M NaCl, and again in water (21). Finally, the polyhedra were

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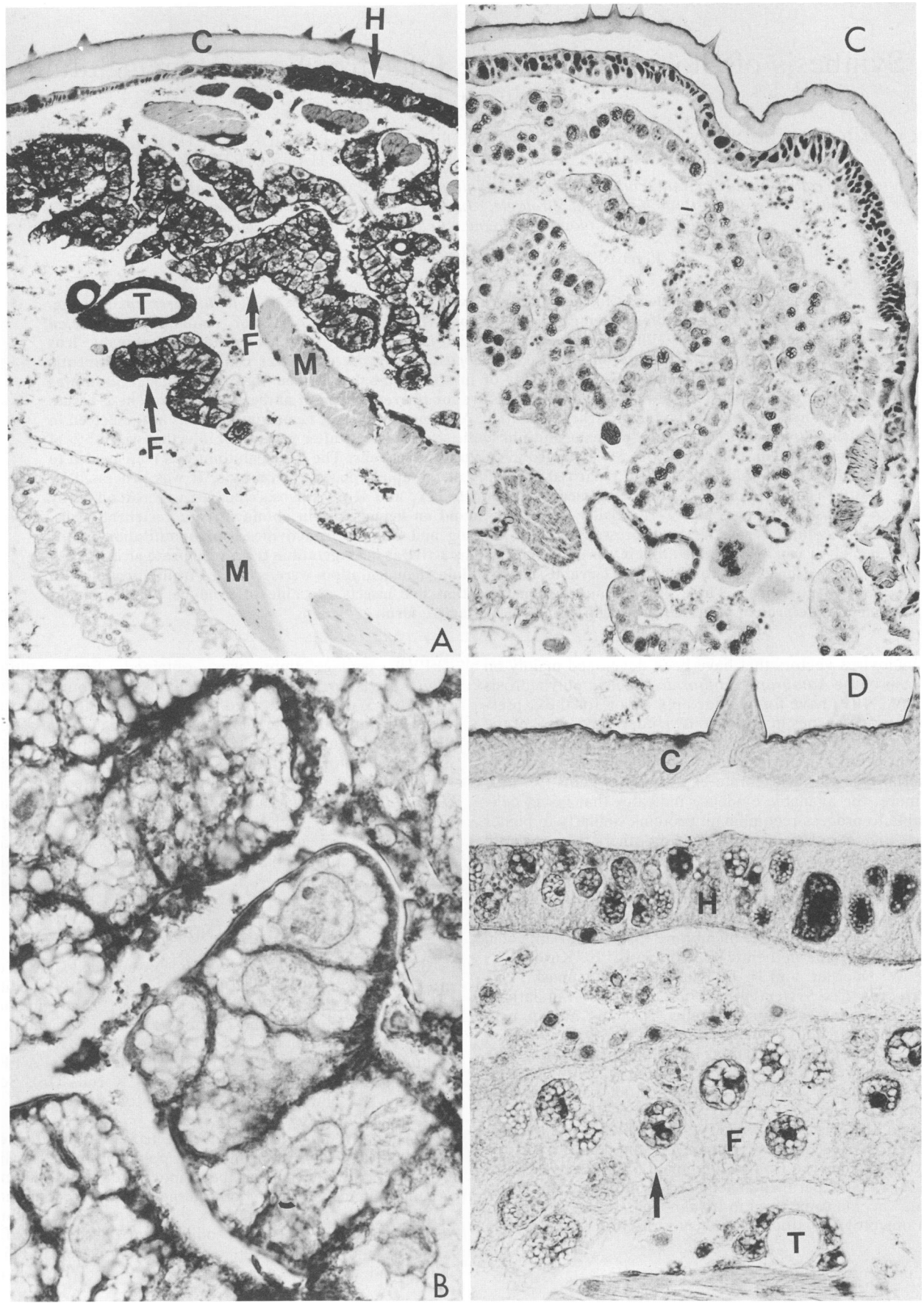
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pelleted through a cushion of 30% (wt/wt) sucrose (100,000 × *g*, 30 min, 4°C), washed with water, and counted in a hemacytometer. For coinfection, various ratios of authentic and recombinant AcNPV were used with multiplicities of infection (MOIs) ranging from 0.1 to 2.0 PFU/cell. When the MOI was 0.1 or 0.2 for authentic and recombinant virus, respectively, conditions were optimal for hemagglutinin expression in larvae of *Heliothis virescens*.

Raising and infection of insects. Eggs from *Heliothis virescens* were disinfected with formaldehyde vapor to avoid inapparent infection of the larvae. Hatching larvae were raised on a semisynthetic diet in groups until they reached the fourth instar stage. For parenteral infection, fourth instar larvae were injected into the hemocoel with 20 μl of cell culture supernatant containing 10^5 to 10^6 PFU of authentic or recombinant AcNPV and raised individually in cups on the semisynthetic diet at 23°C.

For oral infection, fourth instar larvae were placed individually in cups with a piece of food containing 10^6 polyhedra, small enough to be ingested within 24 h. Thereafter, larvae were transferred to cups with fresh food and incubated at 23°C.

Larvae were stripped of their guts to avoid proteolytic degradation and homogenized for 1 min in 0.5 ml of phosphate-buffered saline (PBS) containing protease inhibitors (5,000 U of aprotonin per ml, 1 mM phenylmethylsulfonyl fluoride [PMSF], or 10 mM iodoacetamide) with an Ultra-Turrax blender. After brief low-speed centrifugation, the supernatant and the white upper layer of the pellet were removed from the lower black layer and used for further analyses. For histological studies, intact larvae were fixed in methanol.

Hemagglutination and hemolysis assays. Hemagglutination activity was assayed by the standard titration technique. Hemolysis assays were done as follows. A volume of 200 μl of homogenate was mixed with 200 μl of a 1% suspension of sheep erythrocytes in PBS containing 1% bovine serum albumin and incubated for 15 min on ice. After centrifugation for 3 min at 500 × *g*, the pellet was suspended in 400 μl of 140 mM NaCl–5.4 mM KCl–20 mM MES [2(*N*-morpholino)-ethanesulfonic acid] at the appropriate pH. The mixture was incubated at 37°C for 20 min and centrifuged, and the optical density of the supernatant was measured at 520 nm.

DNA blot hybridization. Virus isolated from polyhedra was suspended in PBS and applied to a nitrocellulose filter in a slot-blot apparatus (Schleicher & Schuell, Düren, Federal Republic of Germany [FRG]). The nitrocellulose filter was placed successively on Whatman 3 MM paper saturated with 0.5 M NaOH, 1 M Tris hydrochloride (pH 7.5), and SSC (0.3 M NaCl–0.03 M sodium citrate) buffer. After baking at 80°C for 2 h, the filter was hybridized with the nick-translated small *Hin*dIII fragment of pUC-HA 651/34 (8) as a hemagglutinin-specific probe and with the small *Xho*I-*Eco*RV fragment of pAc-YM1 (12) as an AcNPV-specific probe.

Immunoblotting. The proteins were blotted from SDS-polyacrylamide gels to nitrocellulose filters as described previously (8). For the quantification of the hemagglutinin, the slot-blot technique was used. Appropriate amounts of homogenate in RIPA buffer (1% Triton X-100, 1% DOC, 0.1% SDS, 0.15 M NaCl, 10 mM EDTA, 1 mM PMSF, 10 mM iodoacetamide, 5,000 U of aprotinin per ml, 20 mM Tris hydrochloride [pH 6.8]) were blotted onto a nitrocellulose filter by using the slot-blot apparatus. The blots were blocked overnight at 4°C in a solution of 10% milk powder in PBS. After being washed with PBS containing 0.1% Tween-20, slot blots were incubated with rabbit anti-HA_2 serum. An anti-FPV serum was used for Western immunoblots. Bound antibody was detected by using biotinylated anti-rabbit immunoglobulin G and streptavidin-biotinylated horseradish peroxidase complex (Amersham-Buchler, Braunschweig, FRG) following the manufacturer's protocol.

Immunohistology. Peroxidase-antiperoxidase method. Infected or uninfected larvae were fixed in methanol at 4°C for at least 24 h. Cross-sections were embedded in paraffin by standard techniques. Hemagglutinin was visualized by the peroxidase-antiperoxidase (PAP) method essentially as described previously (15) with rabbit antiserum prepared against the HA_2 subunit of the hemagglutinin (1) for the primary antibody reaction. The peroxidase activity was monitored with diaminobenzidine. Cell nuclei were counterstained with hematoxylin. Sections not exposed to the PAP technique were stained with hematoxylin and eosin. Sections of mock-infected larvae exposed to hemagglutinin-specific antiserum and sections of infected larvae exposed to preimmune serum were used as controls.

Immunofluorescence. Tissue pieces were rapidly frozen in melting isopentane (cooled with liquid nitrogen) and then carefully freeze-dried and embedded in Epon. Thin tissue sections were mounted on glass slides and processed for immunostaining exactly as described in detail elsewhere (3).

Transmission electron microscopy. Small tissue pieces were fixed with 2% glutaraldehyde in PBS (pH 7.4), post-fixed in 2% osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in Epon. Ultrathin sections were stained with lead citrate and uranyl acetate and viewed with a Zeiss EM10 electron microscope.

Scanning electron microscopy. Tissues were fixed and dehydrated as described above and dried in a critical-point drier (Watford, England). After being coated with gold in a sputtering device (Balzers Union, Balzers, Fla.), samples were analyzed in an ETEC Autoscan scanning electron microscope at 20 kV.

Immunization procedure. For immunization of 30-week-old chickens, larvae obtained 4 days after parenteral infection with recombinant ACNPV were used. Chickens were inoculated once intramuscularly (i.m.) with larval homogenate containing 7 mg of protein and 2,048 hemagglutinating units that was emulsified in Freund complete adjuvant. Control chickens were inoculated with an equal amount of homogenate from larvae infected with authentic AcNPV.

FIG. 1. Tissue tropism of recombinant and authentic AcNPV in *Heliothis virescens* larvae after parenteral infection. (A) Demonstration of hemagglutinin antigen in larvae 4 days after inoculation with recombinant AcNPV. A positive reaction is visible in numerous cells of the fat body (F), in hypodermal cells (H), and in tracheal matrix cells (T). Muscle tissue is negative. (B) Hemagglutinin is present in the cytoplasm of fat body cells, whereas cell nuclei and lipid vacuoles are negative. (C) Tissues of larvae infected with authentic AcNPV are immunohistologically negative for hemagglutinin antigen. Numerous nuclei of the hypodermis and of the fat body are hypertrophic and contain mainly polyhedric inclusion bodies at 4 days after inoculation. Dark staining of nuclei is due to counterstaining with hematoxylin. (D) The same sample as in panel C. Numerous intranuclear polyhedric inclusion bodies are present in hypodermal cells (H), in cells of the fat body (F), and in tracheal cells (T). Note the single cuboidal cytoplasmic inclusion body (arrow). Cytoplasm is negative for hemagglutinin antigen. Thin sections were exposed to hemagglutinin-specific immunohistology by the PAP technique; cell nuclei were counter-stained with hematoxylin. C, Cuticula. Magnification: (A) ×90; (B) ×560; (C) ×90; (D) ×350.

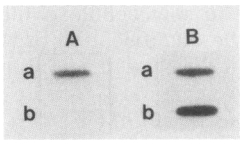

FIG. 2. DNA of recombinant AcNPV in polyhedra purified from doubly infected *Spodoptera frugiperda* cells. Polyhedra were isolated from *Spodoptera frugiperda* cells infected with authentic and recombinant AcNPV (a) or from cells infected with authentic AcNPV alone (b). Virus purified from polyhedra was blotted to a nitrocellulose filter and hybridized with a hemagglutinin-specific (A) or an AcNPV-specific (B) probe.

Two weeks later, the birds were challenged by i.m. infection with 10^4 PFU of FPV. Blood samples were obtained before challenge, and the sera were inactivated at 45°C for 30 min. The hemagglutination inhibition and neutralization tests were performed according to standard procedures.

RESULTS

Entry into and spread of recombinant virus in larvae. In nature, infection of insect hosts by baculoviruses is accomplished by oral ingestion of virions occluded in polyhedra. Following dissolution of the occlusion bodies in the midgut lumen, released virus particles are believed to enter the organism via primary infection of the intestinal epithelial cells. Subsequently infection spreads to other susceptible tissues via the hemocoel. The occlusion bodies play an essential role in the mode of infection, because they apparently protect the virions against the aggressive digestive juices of the enteric tract (4). The recombinant virus per se is not suited for this natural route of infection, because it is unable to synthesize polyhedrin. We have chosen two different approaches to overcome this problem in the present study. First, to bypass the intestinal tract, larvae were infected by parenteral injection. Second, recombinant virus

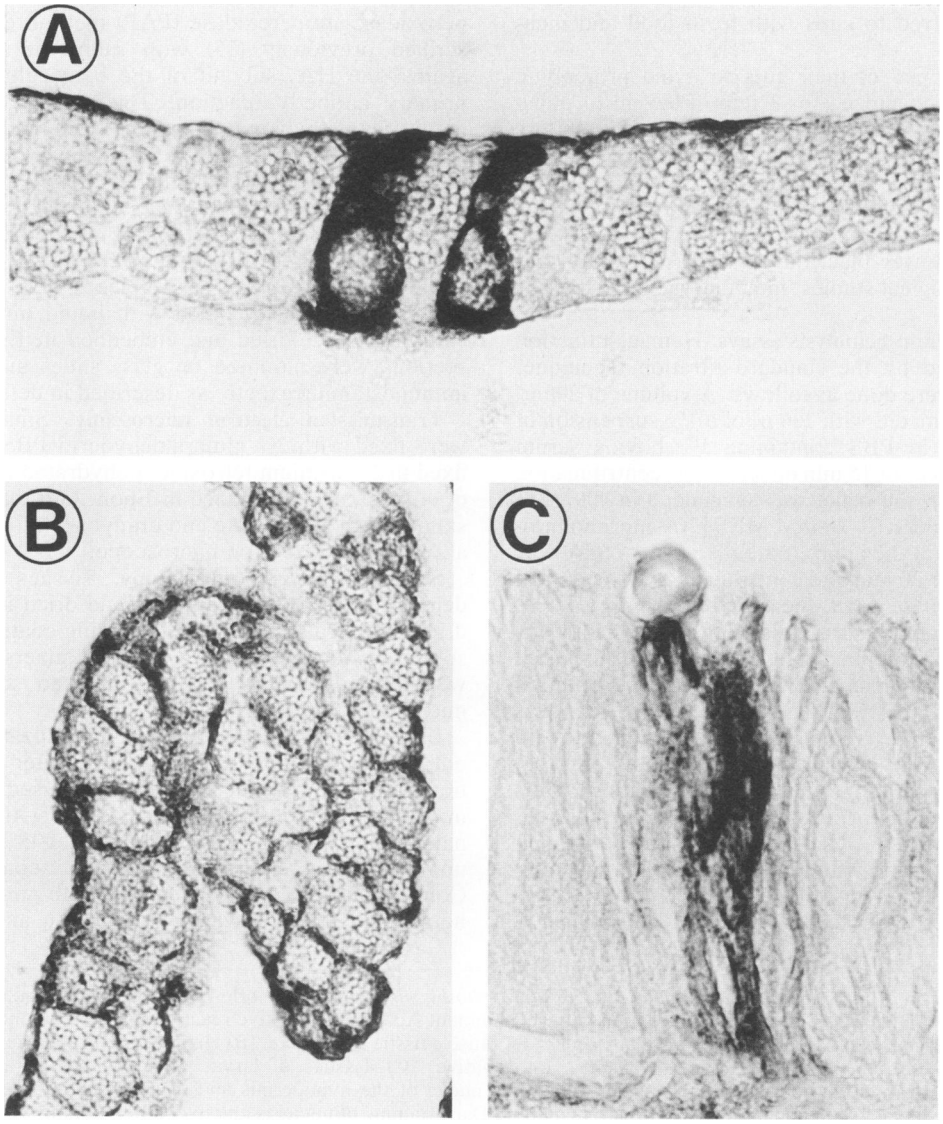

FIG. 3. Tissue tropism of recombinant and authentic AcNPV in *Heliothis virescens* larvae infected per os. (A) Five days after oral double infection, hypodermal cells are hypertrophic and contain numerous polyhedra. Two cells show massive hemagglutinin expression. Polyhedra are hemagglutinin negative. (B) Fat body cells display marginal cytoplasmic hemagglutinin and nuclear polyhedra. (C) A segment of the gut epithelium containing a hemagglutinin-positive cell. Thin sections were stained as described for Fig. 1. Magnification, ×560.

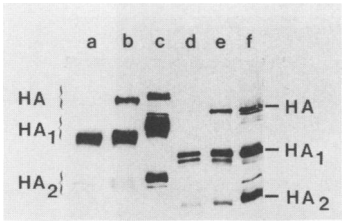

FIG. 4. Processing of hemagglutinin in *Heliothis virescens* larvae and cultured *Spodoptera frugiperda* cells. Larvae were parenterally infected with recombinant AcNPV for 3 days (a, d). *Spodoptera frugiperda* cells were infected with recombinant AcNPV for 3 days (b, e). MDCK cells were infected with FPV overnight (c, f). Suspensions of cells and homogenates of larvae were solubilized with 0.5% Triton X-100. After centrifugation, the hemagglutinin in the supernatant was immunoprecipitated with a mouse monoclonal antibody against FPV hemagglutinin and protein A-Sepharose. The monoclonal antibody (HCl) was a kind gift of A. Hay, National Institute for Medical Research, London, United Kingdom. After being washed with RIPA buffer, the immune complexes were suspended in 50 mM phosphate buffer (pH 7.0) containing 0.1% SDS and 0.5% mercaptoethanol, boiled for 10 min, and briefly centrifuged. The supernatants were incubated at 37°C overnight without (a, b, c) or with (d, e, f) 60 mU of endoglycosidase F (mixture of endoglucosaminidase F and glycopeptidase F) from *Flavobacterium meningosepticum* (Boehringer, Mannheim, Federal Republic of Germany), electrophoresed on a 10% polyacrylamide gel, and then electroblotted to a nitrocellulose membrane. The blot was analyzed with anti-FPV serum from a rabbit as described in Materials and Methods.

was packaged into polyhedra by double infection with wild-type AcNPV and could then be used for infection by natural routes.

Parenteral infection. After parenteral administration, infection with recombinant virus was indistinguishable from control infections with wild-type AcNPV with respect to time course and symptoms of disease. About 3 days after infection, *Heliothis virescens* larvae stopped feeding and died within 6 days, developing the typical symptoms of infection with nuclear polyhedrosis viruses: growth retardation, liquifaction of internal tissues, darkening of the cuticle, and rupture of the epidermis.

To analyze the cell and tissue tropism of recombinant and wild-type viruses, thin sections of infected animals were investigated by immunohistology (Fig. 1). In insects infected with recombinant virus, hemagglutinin could first be detected 2 days postinfection in a few cells of the fat body and hypodermis. By 3 days postinfection, large sections of the fat body expressed hemagglutinin, which was also found in the tracheal matrix. On day 4, there was a further increase in the number of positive cells and in staining intensity but without appearance of the antigen in other organs (Fig. 1A). Hemagglutinin was present in the cytoplasm; nuclei and the lipid vacuoles of the fat body cells were devoid of hemagglutinin (Fig. 1B).

In larvae infected with wild-type virus, hemagglutinin was not made, but cytopathology typical of AcNPV infection was observed (Fig. 1C). At 24 h postinfection, small eosinophilic nuclear inclusions were formed in some cells of the hypodermis and the fat body. At 2 days postinfection, foci of virogenic stroma, relatively large polymorphous nuclear inclusions believed to be the site of virus assembly (4), could be detected in numerous cells of the fat body, hypodermis, and tracheal matrix. At the same time, polyhedral occlusion bodies appeared in these tissues and steadily increased in number during the following days. Frequently, there were also cuboidal cytoplasmic inclusions of various sizes. Formation of occlusion bodies was paralleled by hypertrophy of the nuclei and, in the hypodermal cells, also of the cytoplasm (Fig. 1D). Virogenic stroma was formed with the same tissue distribution and the same time dependence in larvae infected with recombinant virus. However, polyhedra and cuboidal cytoplasmic inclusions were never observed under these conditions.

Oral infection. For the reasons outlined above, virus encapsidated into polyhedra was used for oral infection. Occluded recombinant virus was obtained by coinfection with wild-type virus in *Spodoptera frugiperda* cell cultures. The polyhedra were purified from these cells, and the occluded virus was analyzed for the presence of hemagglutinin-encoding DNA by the slot-blot technique (Fig. 2). Whereas DNA obtained from polyhedra after infection with wild-type AcNPV alone hybridized only with a DNA probe complementary to the 3′-flanking region of the polyhedrin promoter, polyhedral DNA from doubly infected cells also had homology to a hemagglutinin-specific cDNA probe. Thus, recombinant virus had been occluded in polyhedra after double infection.

The tissue tropism of infection after oral entry of the virus was also investigated by histological analysis. Hemagglutinin could first be detected 4 days postinfection in the cytoplasm of some cells of the fat body, the hypodermis, and the tracheal matrix. These cells increased in number at days 5 and 6 (Fig. 3A and B). Hemagglutinin was also expressed after oral infection in a few isolated gut cells (Fig. 3C). In general, the number of cells synthesizing hemagglutinin was significantly lower than after parenteral infection. Hypodermis, fat body, and trachea also displayed enlarged cell nuclei, virogenic stroma, and cuboidal cytoplasmic inclusions, again with a delay of about 2 days compared with parenterally infected larvae. Because of the coinfection with authentic AcNPV, hemagglutinin was found together with polyhedra in infected tissues (Fig. 3A and B).

Processing and biological activities of hemagglutinin expressed in *Heliothis virescens*. Hemagglutinin expressed in insects infected via the parenteral route was analyzed by the Western blotting technique. Both the precursor HA and the cleavage products HA_1 and HA_2 were present (Fig. 4). Thus, cleavage had occurred in the larvae. As also apparent from Fig. 4, cleavage was more efficient than in cultured insect cells. The molecular weights of the polypeptides were similar to those of hemagglutinin expressed in *Spodoptera frugiperda* cells but lower than those of hemagglutinin synthesized in vertebrate cells. The differences in molecular weight disappeared, however, after carbohydrate removal by glycopeptidase F treatment. These observations support the concept that hemagglutinin expressed in insect cells has truncated oligosaccharides (Kuroda et al., in press).

To see whether hemagglutinin was transported to the cell surface, larval tissues were exposed to chicken erythrocytes and analyzed for hemadsorption. At 3 to 4 days after infection, attachment of erythrocytes could be detected in a stereomicroscope and by scanning electron microscopy (Fig. 5). Transmission electron microscopy of ultrathin sections revealed close apposition of the erythrocyte and the insect cell membrane, indicating that functional hemagglutinin was present in a relatively large area of the cell surface (Fig. 5, insert). Mock-infected larvae and larvae infected with wild-type AcNPV did not show hemadsorption. Cell surface expression of the hemagglutinin was also analyzed by immunofluorescence microscopy of thin sections of larval tissues. With this technique, hemagglutinin could be clearly detected

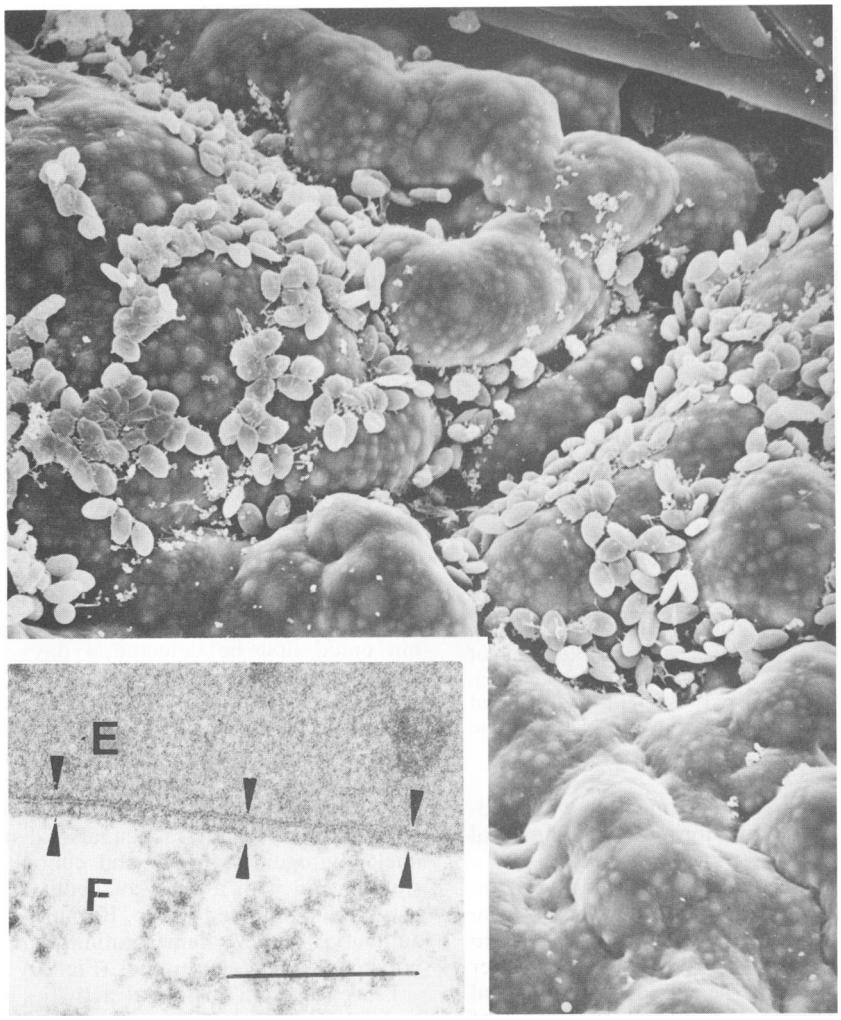

FIG. 5. Hemadsorption of fat body cells of *Heliothis virescens* larvae after infection with recombinant AcNPV. Four days after parenteral infection, larvae were dissected, washed once with PBS, and incubated for 30 min on ice in a 1% suspension of chicken erythrocytes. After removal of nonadsorbed erythrocytes by extensive washes with PBS, samples were processed for electron microscopy as described in Materials and Methods. The scanning electron micrograph (magnification, ×400) shows numerous erythrocytes attached to the surfaces of fat body cells. Inset: Thin section showing the site of close attachment between a chicken erythrocyte (E) and a fat cell (F). Arrowheads indicate the adjoining lipid bilayers. Bar, 0.2 μm.

on the apical surface of tracheal cells but proved to be absent on the basolateral plasma membrane (Fig. 6). Thus, hemagglutinin can be expressed in a polarized fashion in insect cells.

After parenteral infection with recombinant virus, hemagglutinating activity could be detected in homogenized larvae at 2 days and reached a maximal level at 3 to 4 days postinfection (Fig. 7A). Hemagglutinin expression was delayed by 3 days and hemagglutinin titers were lower when the virus was administered by the oral route (Fig. 7B). Low hemagglutination titers, presumably due to lipids, were also observed occasionally in larvae infected by authentic virus and in mock-infected larvae. However, unlike the hemagglutination occurring after infection with recombinant virus, this activity could not be inhibited by an FPV-specific antiserum. The level of hemagglutinating activity observed after parenteral infection was similar to that found in FPV-infected MDCK cells (ca. 1,000 HAU/mg of protein). The amount of hemagglutinin expressed in the larvae was also determined by a slot-blot assay with HA_2-specific antiserum

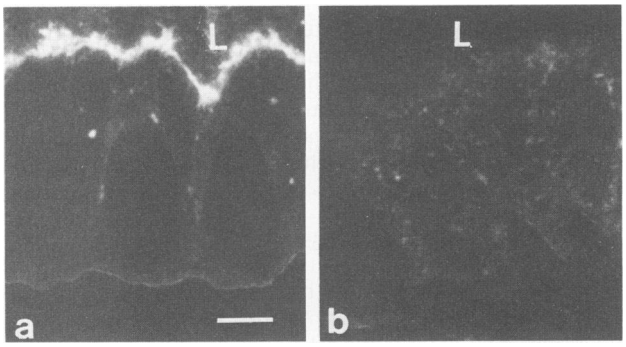

FIG. 6. Polarized expression of hemagglutinin at the apical surface of tracheal epithelium. Hemagglutinin was localized by immunofluorescence labeling in tissue sections of the tracheal epithelium 4 days after parenteral infection. (a) Animals infected with recombinant AcNPV; (b) animals infected with wild-type AcNPV. Note polarized distribution of hemagglutinin along the apical plasma membrane that borders on the tracheal lumen (L). Bar, 5 μm.

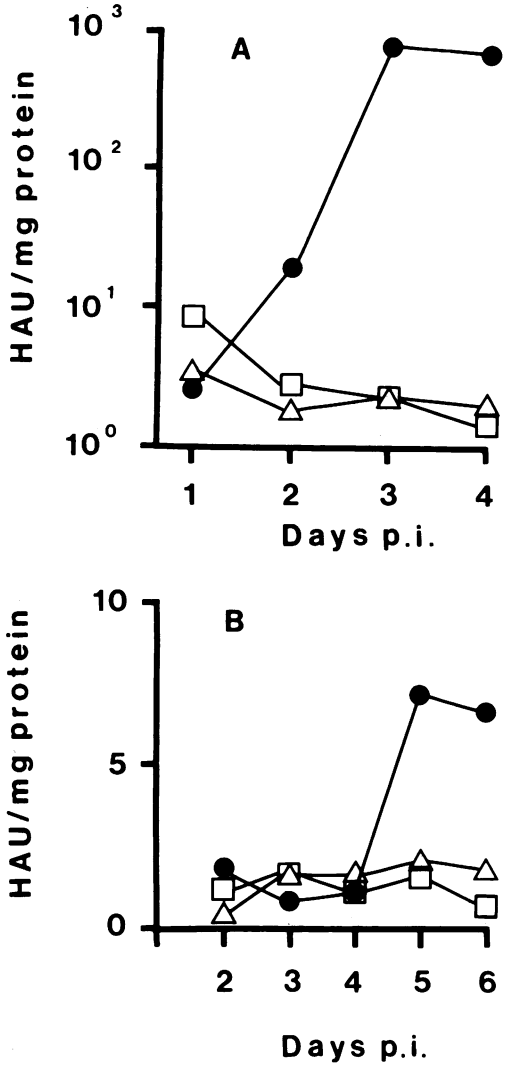

FIG. 7. Production of hemagglutinin in *Heliothis virescens* larvae. Larvae were infected parenterally (A) or per os (B) with recombinant AcNPV (●), authentic AcNPV (△), or mock infected (□). Hemagglutinin activity was analyzed in homogenized larvae. p.i., Postinfection.

and purified FPV for comparison. Compared with MDCK and *Spodoptera frugiperda* cell cultures, in which the hemagglutinin yields were 2 and 5% of total protein, respectively, hemagglutinin yields reached about 0.3% of total larval protein after parenteral infection (Fig. 8). After oral infection the yields were below 0.1% (data not shown).

The influenza virus hemagglutinin induces hemolysis at low pH. This activity, which has already been observed on hemagglutinin obtained from *Spodoptera frugiperda* cells (8), was also found after expression in insect larvae. Inhibition by antisera raised against influenza virus indicated the hemagglutinin-specific nature of this activity (Fig. 9). For a quantitative comparison of hemagglutinin expressed in insect and in vertebrate cells, fusion activity was correlated to hemagglutinin amounts determined by slot-blot assay (Fig. 10). When hemagglutinin from insect cell cultures was compared with MDCK cell-derived hemagglutinin, it was found that almost identical amounts were needed to achieve a given extent of hemolysis. However, about two to four times

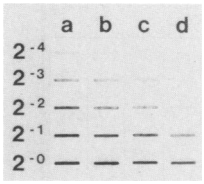

FIG. 8. Hemagglutinin yields obtained from insect cells. Serial dilutions of (a) purified FPV (containing from 25 to 400 ng of hemagglutinin), (b) homogenates of FPV-infected MDCK cells (1 to 16 μg of protein), (c) homogenates of *Spodoptera frugiperda* cells infected with recombinant AcNPV (0.225 to 3.6 μg of protein), and (d) homogenates of larvae infected parenterally with recombinant AcNPV (3.9 to 62.5 μg of protein) were applied to a nitrocellulose filter. The blot was incubated with antihemagglutinin serum. Bound antibody was detected as described in Materials and Methods.

as much hemagglutinin was required when it was obtained from larvae.

To test the immune response to the hemagglutinin, chickens were immunized with homogenates of larvae as de-

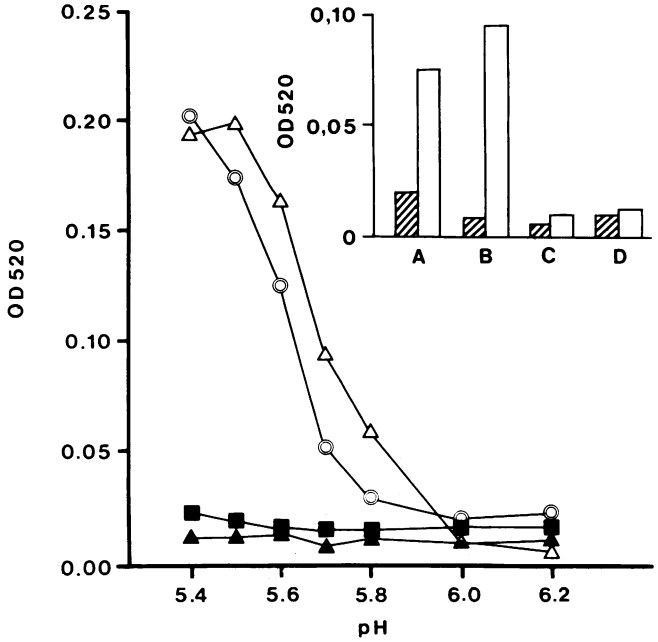

FIG. 9. Hemolytic activity of hemagglutinin expressed in *Heliothis virescens* larvae. Larvae were parenterally infected with recombinant (○) or authentic (■) AcNPV or were mock infected (▲). At 3 days after infection, the larvae were homogenized, and samples containing 120, 170, and 190 μg of protein from recombinant-AcNPV-infected, authentic-AcNPV-infected, and mock-infected larvae, respectively, were assayed for hemolytic activity at the pHs indicated. Homogenates of recombinant-AcNPV-infected *Spodoptera frugiperda* cells containing 40 μg of protein were also analyzed for hemolytic activity (△). The slight difference in pH optimum of hemolytic activity observed between hemagglutinin from larvae and *Spodoptera frugiperda* cells is not considered significant, because it was not seen in other experiments. (Inset) Inhibition of hemolytic activity by an anti-FPV rabbit serum. Homogenates of recombinant-AcNPV-infected *Spodoptera frugiperda* cells (A), recombinant-AcNPV-infected larvae (B), authentic-AcNPV-infected larvae (C), and mock-infected larvae (D) were incubated with (hatched bars) or without (open bars) anti-FPV serum for 1 h on ice, mixed with sheep erythrocytes, and assayed for hemolytic activity at pH 5.5.

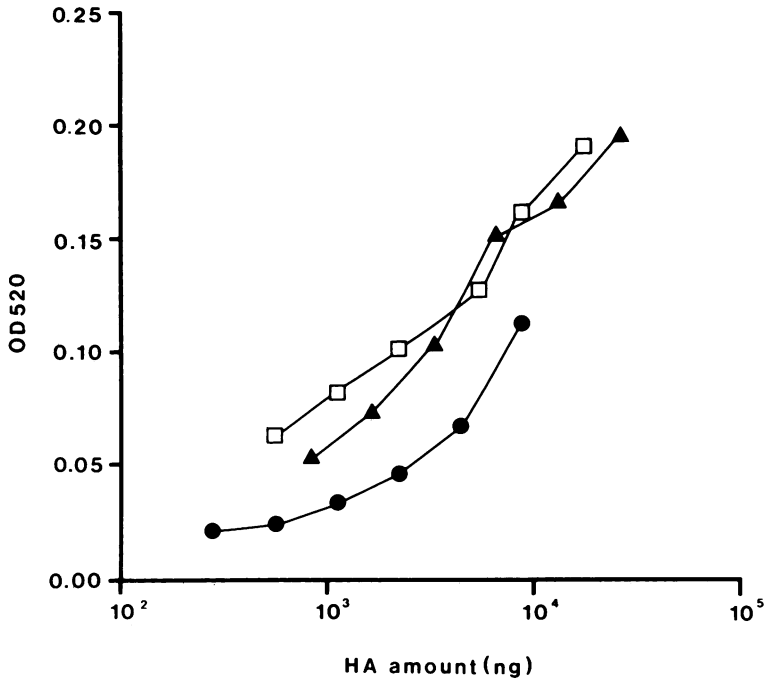

FIG. 10. Dose dependence of hemolytic activity. The hemolytic activity of hemagglutinin from recombinant-AcNPV-infected larvae (●), recombinant-AcNPV-infected *Spodoptera frugiperda* cells (▲), and FPV-infected MDCK cells (□) was analyzed. Homogenates of larvae or cultured cells containing the indicated amounts of hemagglutinin were mixed with sheep erythrocytes, and the hemolytic activity was measured at pH 5.5. The amount of hemagglutinin was determined by the slot-blot technique described in Materials and Methods.

scribed in Materials and Methods. The immunized animals produced hemagglutination-inhibiting antibodies at titers of 1:8 to 1:16 and survived a challenge infection with 10^4 PFU of FPV without showing signs of fowl plague, while the control animals immunized with wild-type AcNPV-infected larvae died at 2 days postinfection.

DISCUSSION

Live insects have been considered a possible alternative to cell cultures for mass production of recombinant proteins via baculovirus vectors. Despite the fact that a large variety of different proteins have already been obtained from cell cultures, the insect system has so far been used only for the expression of secretory proteins (5, 10, 11, 13). We describe here production of a recombinant membrane protein in live insects.

As an alternative to inoculation through injection, which is a rather tedious and time-consuming procedure, larvae have also been infected by the natural route of oral ingestion, which may be more suitable for mass production. We show here that this can be accomplished when recombinant virus is occluded in polyhedra obtained from doubly infected cell cultures. As indicated by DNA hybridization, polyhedra found in larvae infected by the latter procedure also contain recombinant virus (data not shown). Thus, vertical transmission of recombinant virus within a larval population appears to be possible under these conditions and may provide the means for permanent production of the hemagglutinin or any other desired protein.

Hemagglutinin synthesis has been found to occur in several tissues, notably in the fat body, hypodermis, and trachea, but not in others, such as muscle. The observation that individual gut cells expressed hemagglutinin after oral infection is compatible with the view that this organ is the site where primary infection takes place under these conditions of virus entry. It is interesting, however, that after parenteral infection hemagglutinin-producing gut cells were not detected. This finding might indicate that these cells can be infected from the apical but not from the basolateral side. It should be pointed out that the tissues expressing hemagglutinin were identical with the tissues containing polyhedra after infection with wild-type virus or after double infection with recombinant and wild-type viruses. Thus, it is fair to conclude that, like wild-type AcNPV, the recombinant virus is restricted in its host range to insect cells and does not represent a potential biohazard for vertebrates.

The amount of hemagglutinin obtained from *Heliothis virescens* larvae infected parenterally was about 0.3 mg/100 mg of protein, whereas tissue cultures yielded 5 mg/100 mg of protein. The fact that some larval tissues are unable to produce hemagglutinin may account for this difference. It should be worthwhile testing whether the hemagglutinin yields obtained after oral infection, which were almost 1 order of magnitude lower and therefore have to be considered not satisfactory for biotechnological exploitation, could be enhanced by improving experimental conditions, such as selecting new vectors and other host systems.

The data presented here show that the hemagglutinin is processed in *Heliothis virescens* tissues basically in the same fashion as in cultures of *Spodoptera frugiperda* cells. The protein undergoes posttranslational proteolytic cleavage at an arginine-containing cleavage site, which is necessary for its fusion activity. As is the case in *Spodoptera frugiperda* cells, the hemagglutinin obtained from *Heliothis virescens* had lower electrophoretic mobility than hemagglutinin from vertebrate cells, indicating that the oligosaccharide side chains have been trimmed but not elongated to the complex type (Kuroda et al., in press). These observations imply that

hemagglutinin is processed on cellular membranes by translocation in the endoplasmic reticulum, removal of the signal sequence, and transport to the Golgi apparatus. The hemagglutinin is then further transported to the cell surface. This was shown by hemadsorption as well as immunofluorescence labeling. We also demonstrate here that the hemagglutinin is expressed in tracheal epithelium exclusively in the apical plasma membrane, as it is in polarized vertebrate cells, such as MDCK cells (17). This observation indicates that insect cells possess sorting mechanisms to maintain plasma membrane polarity similar to vertebrate cells and that the influenza virus hemagglutinin is recognized in insect cells by this machinery.

As a consequence of correct processing, the hemagglutinin derived from *Heliothis virescens* displays all of the biological activities of authentic FPV hemagglutinin. It induces hemadsorption, causes hemolysis, reacts with hemagglutin-specific antibodies, and protects animals from infection with FPV. The quantitative analysis of the hemolytic activity performed in this study indicates that the functional fraction of total hemagglutinin is about half as large in larvae as it is in vertebrate or insect cell cultures. Unpublished studies with gradient centrifugation have revealed that larvae and MDCK cells contain both the monomeric and the trimeric forms of the hemagglutinin. However, there is a prevalence of monomers over trimers in larvae which is not observed in MDCK cells. Thus, defective assembly of hemagglutinin spikes may be one reason for the reduced biological activity. Other factors that might be important are the sizes of the membrane vesicles carrying the hemagglutinin or exposure of the hemagglutinin on these vesicles. In any case, the reduction in the biological activity appears to be too small to seriously invalidate *Heliothis virescens* larvae as an expression system for the influenza virus hemagglutinin.

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