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Complementation of recombinant baculoviruses by coinfection with wild-type virus facilitates production in insect larvae of antigenic proteins of hepatitis B virus and influenza virus

(baculovirus/hepatitis B surface antigen/influenza A virus neuraminidase)

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ABSTRACT **We describe the coinfection of insects with wild-type and recombinant baculoviruses in which the polyhedrin gene promoter is used to express hepatitis B virus envelope protein (hepatitis B virus surface antigen; HBsAg) or influenza A virus neuraminidase (NA). Viruses were administered *per os* to larvae of the cabbage looper, *Trichoplusia ni*, causing an infection that within 5 days resulted in the production of ≈0.15 mg of HBsAg per insect, representing 1.5% of the total extracted protein, or ≈2.8 mg of NA per insect, representing 28% of the total extractable protein. The HBsAg and NA produced by infected larvae were purified from insect lysates. These proteins were antigenic as determined by conformation-dependent immunoassays. The NA was enzymatically active with conventional substrates. The method of infection described allows genetic complementation by wild-type virus of recombinant viruses lacking the polyhedrin gene essential for infection *per os* and has implications for the high-yield production in insect larvae of other recombinant proteins of baculoviruses.**

Infection of insect cells *in vitro* or insect larvae *in vivo* with baculovirus results in the production by virus of polyhedra, polymers of the 33-kDa polyhedrin protein (1, 2). The polyhedrin protein is overexpressed *in vivo* and in cultured cells can eventually account for up to 75% of the total cellular protein. The polyhedra enclose the newly synthesized virus particles and facilitate *in vivo* horizontal transmission of the virus, primarily by preventing virus inactivation in the environment and in the insect gut after ingestion. After the infection is initiated by entry of the virus into epithelial cells of the midgut region, the polyhedra are no longer necessary to perpetuate the infection (3). Because the polyhedrin protein is not required for *in vitro* infection (4), a foreign gene can be substituted for the structural gene for polyhedrin without affecting viral replication and thus can then utilize the efficient polyhedrin promoter. This system has been used effectively for the expression of many recombinant proteins (for review, see ref. 5). Although the activity of the polyhedrin promoter is very high in cultured cells, it is even higher in insect larvae (6). Therefore, it seems desirable to utilize larval infection for the efficient and inexpensive production of baculovirus recombinant proteins. However, because recombinant viruses lack polyhedron encapsidation, the efficiency of infection of larvae by the oral route is low. Although nonencapsidated recombinant baculoviruses may be injected directly to circumvent the requirement for encapsidation (7), this method of infection is technically difficult and occasions high mortality in inoculated larvae.

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In the present paper, we describe a strategy to increase the *in vivo* infection rate of recombinant viruses and we demonstrate its efficiency in producing high yields of two foreign viral proteins, hepatitis B virus (HBV) surface antigen (HBsAg) and influenza A virus neuraminidase (NA). This stratagem involves complementation through dual infection of recombinant and wild-type baculoviruses to produce encapsidated recombinant virus particles that readily infect *Trichoplusia ni* larvae when given by the oral route.

MATERIALS AND METHODS

Viruses. The wild-type baculovirus, *Autographa californica* nuclear polyhedrosis virus, was used in coinfection experiments with two recombinant viruses. One recombinant had been constructed by replacing the polyhedrin gene with a gene for HBsAg (8). The HBsAg gene was isolated from a clone of HBV genome derived from the *ayw* subtype. The influenza virus NA gene was isolated from the pNASV2330 plasmid that codes for the NA of A/Udorn/72 (H3N2) virus (9). This plasmid was generously provided to us by C.-J. Lai (Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases).

Infection of Larvae. Cells in monolayer culture of *Spodoptera frugiperda* were infected with multiplicity ratios of 10:1, 1:1, or 1:10 of wild-type and recombinant virus. Five days after infection, virus from the coinfected culture was collected from medium and used to infect fourth instar larvae of the cabbage looper (*Trichoplusia ni*). The larvae had been maintained on a semisynthetic diet and kept at 28°C and 60% relative humidity. After they had molted to become fourth instar larvae, the insects were removed from the diet and starved for 7 hr. The insects then were placed in contact with the virus-containing insect cell culture medium containing a dosage equivalent to the LD_{95} of the wild-type virus (10). After exposure to virus for 1 hr, the larvae were placed on a fresh semisynthetic diet and kept as before. At various times postinfection, moribund larvae were collected and frozen at −70°C.

Recombinant Protein Isolation. Ten volumes of NME buffer (150 mM NaCl/25 mM Mes, pH 7.0/1 mM EDTA/0.1 mM 2-mercaptoethanol/0.02% NaN_3) was added to ≈100 mg of insect (wet weight) and sonicated on ice for 45 sec. Triton X-100 was added to 0.5% and the suspension was centrifuged at 16,000 × *g* for 20 min. The lipid layer was removed by aspiration and the supernatant was collected. Protein concentrations were measured with a Bio-Rad protein kit. HBsAg was determined with a solid-phase RIA (Connaught Laboratories) and plotted with respect to days after infection

Abbreviations: HBV, hepatitis B virus; HBsAg, HBV surface antigen; NA, neuraminidase; CFA, complete Freund's adjuvant.

and the original multiplicity ratios used to infect the tissue culture cells (Fig. 1). The presence of NA protein was determined by assaying for neuraminidase activity by employing fetuin (11) and synthetic [2′-(4-methylumbelliferyl)-α-D-*N*-acetyl neuraminic acid] substrates (12). HBsAg was purified from the supernatant from an insect larval lysate after a 1:5 dilution with NME buffer and adjustment to a density of 1.2 g/ml by addition of 230 mg of CsCl per ml. The sample was centrifuged in a Ti 70.1 rotor at 46,000 rpm for 42 hr at 5°C. Fractions were collected, and those containing maximum HBsAg activity (shaded area, Fig. 2) were pooled and dialyzed against NME buffer. For the purification of NA, the insect larval lysate was chromatographed on DEAE-Sephadex according to Gallagher *et al.* (13) with the modification that the eluent was dialyzed against buffer containing 20 mM $CaCl_2$ (22).

Protein Electrophoresis and Electrophoretic Transfer (Western) Blotting. Aliquots from dialyzed putative HBsAg (Fig. 2) were subjected to electrophoresis on 15% polyacrylamide gels (14) and either stained for protein (Bio-Rad silver stain) or transferred to nylon (15, 16). The nylon was blocked for 16 hr with 5% nonfat dry milk, TBST (10 mM Tris, pH 8.0/150 mM NaCl/0.05% Tween 20), 0.1% NaN_3, and 2 μg of bovine IgG per ml. The primary antibody (monoclonal anti-HBsAg, a gift from L. Mimms, Abbott) was diluted into TBST and incubated with the nylon for 16 hr. After washing, the antibody was detected with an alkaline phosphatase-coupled secondary antibody (Promega Biotec).

Serologic Methods. Twenty 10-week-old female BALB/c mice were injected with 10 μg of HBsAg isolated from the peak fraction of a CsCl gradient (Fig. 2). The serum was tested 2 weeks later for anti-HBsAg antibodies. The procedure of Khan *et al.* (17) was adapted for measuring ELISA anti-HBsAg titers. Microtiter plate wells were coated with 40 ng per well using HBsAg (purified from a chronic carrier patient) prior to applying the mouse serum. The mouse serum was diluted from 1:300 up to 1:24,300 using 0.15 M phosphate-buffered saline, pH 7.4/0.05% Tween 20 (PBS/Tween) containing 2% tissue culture medium from wild-type baculovirus-infected cells. The binding of mouse antibody to the HBsAg was determined using alkaline phosphatase-conjugated sheep anti-mouse immunoglobulin.

The NA activity of the insect lysates were assayed for specific inhibition in a NA inhibition test (18). Specific inhibition of NA activity was demonstrated with mixed antisera from rabbits immunized with purified NA isolated from reassortant viruses deriving their NA from either A/Aichi/2/68 or A/Leningrad/360/86.

RESULTS

Production of HBsAg. The yield of HBsAg per insect increased if older insects were infected, although this was a function of the increased weight of the insect; the kinetics of production of HBsAg were similar. Whether day-old (neonate), 3-day-old second instar, or 6-day-old fourth instar larvae were infected with a 1:1 ratio of viruses, a plateau of 12.8–16.2 μg of HBsAg per 100 mg of insect was synthesized after 3 days. The production of HBsAg by the larvae was increased by increasing the ratio of recombinant to wild-type virus used in the coinfection in culture (Fig. 1). Infection of larvae with virus produced by cultured cells that had been coinfected with a 10:1 ratio of recombinant:wild-type virus resulted in HBsAg production of 60 μg/100 mg of insect 5 days after infection, at which point all of the insects were harvested. This represented 160 μg of HBsAg per larva using fourth instar larvae. Conversely, infection of larvae with a 1:10 ratio of recombinant:wild-type virus resulted in a lower level of HBsAg production, the maximum expression being 8 μg/100 mg of insect 4 days after infection.

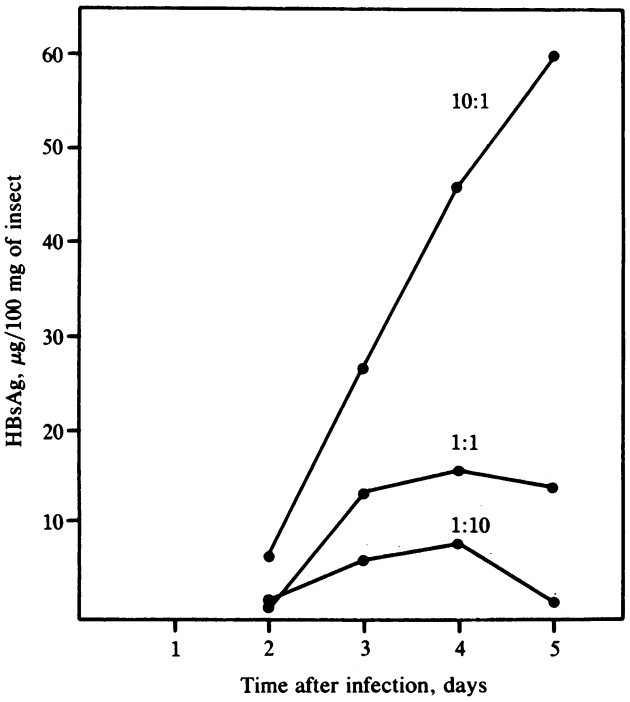

FIG. 1. Kinetics of HBsAg production by insect larvae at different ratios of recombinant:wild-type virus.

The density (1.2 g/ml, Fig. 2) and morphologic appearance (data not shown) of the recombinant HBsAg were identical to that of HBsAg isolated from the serum of infected patients. In addition, an immunoblotted sodium dodecyl sulfate/polyacrylamide gel of recombinant HBsAg isolated from a CsCl gradient (Fig. 3, lanes 3 and 5) demonstrated the presence of unglycosylated (24 kDa) and glycosylated (27 kDa) forms of sAg. This is a similar profile to that produced by

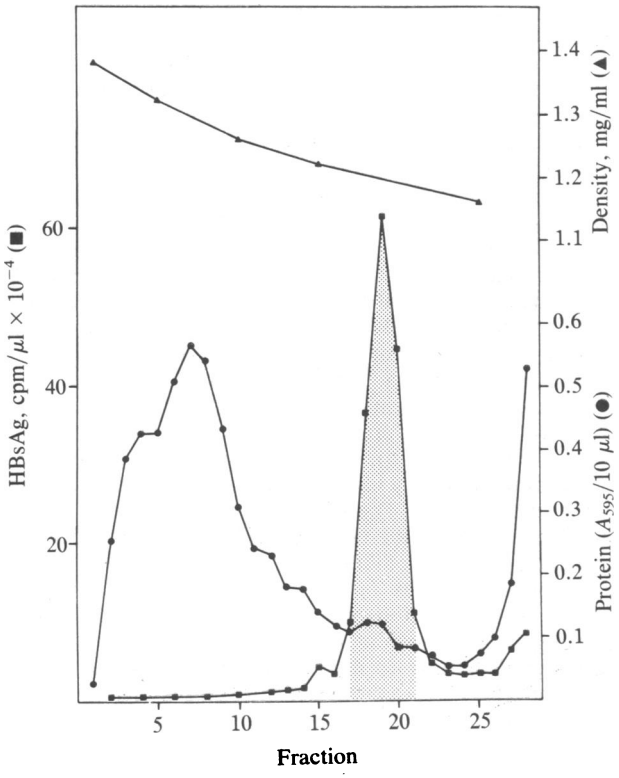

FIG. 2. CsCl purification of insect HBsAg. The shaded area containing maximum HBsAg was dialyzed and pooled.

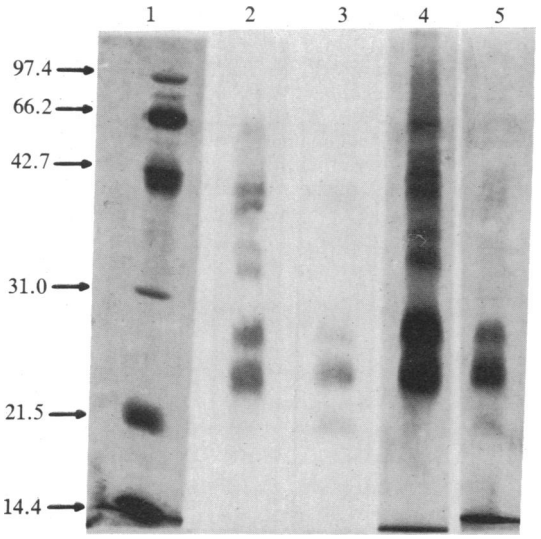

FIG. 3. Immunoblot and silver stain of HBsAg protein. Lanes 1, 4, and 5 were silver stained; lanes 2 and 3 were immunoblotted. Lane 1, molecular mass markers (sizes shown in kDa); lanes 2 and 4, 200 ng of HBsAg purified from the serum of a chronic carrier; lanes 3 and 5, 150 ng of HBsAg produced in insect larvae and prepared as described in the text. Major species of proteins that are immunoreactive with anti-HBsAg are present at 24 and 27 kDa. An immunoreactive protein at 21 kDa is most likely the result of specific protease cleavage, as has been noted to occur in HBV envelope proteins (19, 20).

infected insect cells *in vitro*, although the larger molecular weight forms of HBsAg formed by initiation of translation at upstream sites in infected cultured cells (8) were not detected. Also, the 24-kDa and 27-kDa species are the same as those of HBsAg isolated from the serum of HBV carriers (Fig. 3, lanes 2 and 4; ref. 21). Also evident in the serum-derived HBsAg are the GP42 and P39 and the GP36 and GP33 proteins, high molecular weight forms of HBsAg produced by upstream initiation of translation containing either 174 or 55 additional amino acids, respectively.

The immunogenicity of the insect larval HBsAg was determined by injection of 10 μg of CsCl gradient-purified sAg into mice with and without complete Freund's adjuvant (CFA). Two weeks after infection, the primary ELISA anti-HBsAg serum antibody titer (17) of five mice injected without CFA ranged from 2128 to 3068 (average, 2613), and the antibody titer of five mice injected with CFA ranged from 12,394 to 36,782 (average, 23,067). At this time, the mice were injected with 1 μg of HBsAg, and antibody levels were determined after another 7 days. The five mice originally not injected with CFA developed HBsAg antibody titers ranging from 4050 to 7207 (average, 5165), and those originally injected with CFA developed titers ranging from 30,600 to 156,214 (average, 106,874). These values demonstrate primary and secondary response to the antigen, evidence that the HBsAg is immunogenically active.

Production of Influenza Virus NA. Infection of larvae with a ratio of 10:1 NA-recombinant:wild-type virus resulted in production of 19, 134, and 1120 μg of NA per 100 mg of insect after 3, 4, and 5 days of infection, respectively. This represents a maximum expression of 2.8 mg of NA per insect, or 28% of the total extractable protein. Insect larvae infected with recombinant baculovirus containing the NA gene produced functional NA, as evidenced by the NA enzymatic activity of insect lysates on fetuin and artificial substrates. This activity was inhibited by N2-specific polyclonal antisera (Fig. 4); therefore, the NA made by insects infected with recombinant baculovirus is antigenically equivalent to NA found in intact influenza A virus.

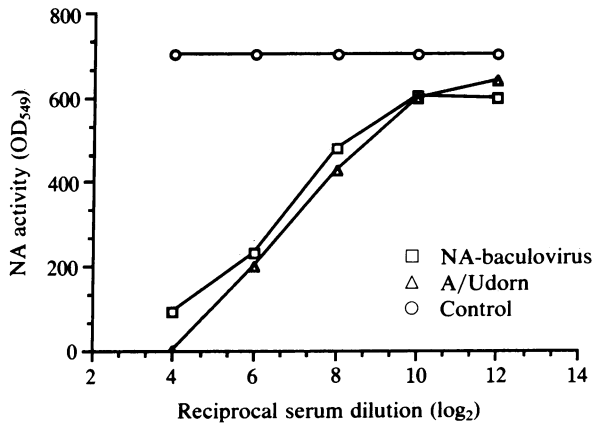

FIG. 4. NA inhibition test. Control represents NA levels without antiserum. NA isolated from influenza A/Udorn and recombinant baculovirus-infected larvae was titrated against increasing dilutions of N2-specific antiserum.

DISCUSSION

The present study has demonstrated that simultaneous coinfection of *Spodoptera frugiperda* cells with recombinant and wild-type baculoviruses results in complementation to provide the recombinant virus with a polyhedron coat essential for its infectivity *in vivo*. By using this method, we have obtained high levels of expression of glycoprotein antigens of two mammalian viruses. By the physical, chemical, and immunological criteria employed in their analyses, these proteins, HBsAg and influenza A virus NA, are indistinguishable from antigens derived from intact hepatitis B and influenza A viruses.

It is clear that the coinfection technique should enable and facilitate the production of proteins from other baculovirus recombinants that lack a functional polyhedrin gene.

We have not studied the physical mechanism by which the infectivity of recombinant baculoviruses (lacking the polyhedrin gene) is enhanced by simultaneous infection with wild-type virus. However, it is highly probable that complementation to supply the polyhedrin gene function missing in the recombinant results in phenotypic mixing to provide the polyhedron coat for recombinant virions.

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