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Synthesis of the Membrane Fusion and Hemagglutinin Proteins of Measles Virus, Using a Novel Baculovirus Vector Containing the β-Galactosidase Gene

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An improved baculovirus expression vector was developed to expedite screening and facilitate oligonucleotide-directed mutagenesis. This vector contained twin promoters derived from the P10 and polyhedrin genes of *Autographica californica* nuclear polyhedrosis virus. The P10 promoter directed the synthesis of β-galactosidase, whereas the polyhedrin promoter controlled the synthesis of foreign gene products. These two genes recombined with wild-type virus genome to yield recombinants which were polyhedrin negative, produced the foreign gene product, and formed blue plaques when β-galactosidase indicator was present in the agarose overlay. An origin of replication derived from M13 or f1 bacteriophage was also included in the plasmid to permit the synthesis of single-stranded DNA. This template DNA was used to introduce or delete sequences through the process of site-specific mutagenesis. The measles virus virion possesses a membrane envelope which contains two glycoproteins: the hemagglutinin (H) and membrane fusion (F) proteins. The H polypeptide has receptor-binding and hemagglutinating activity, whereas the F protein mediates virus penetration of the host cell, formation of syncytia, and hemolysis of erythrocytes. Genes for these two glycoproteins were inserted into the *Nhe*I cloning site of the modified expression vector described above. The vector and purified wild-type viral DNA were introduced into Sf9 insect cells by calcium phosphate precipitation. A mixture of wild-type and recombinant virus was generated and used to infect Sf9 cells, which were subsequently overlaid with agarose. After 3 days, 0.1 to 1% of the plaques became blue in the presence of β-galactosidase indicator. At least 70% of these blue viral colonies contained the foreign gene of interest as determined by dot blot analysis. Recombinant virus was separated from contaminating wild-type virus through several rounds of plaque purification. Insect cells were then infected with the purified recombinants, and synthesis of H and F proteins was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblot detection and Coomassie blue staining. Glycosylation of the proteins appeared to be impaired somewhat, and the precursor to the F protein was not completely cleaved by the proteases present in insect host cells. On the other hand, both proteins appeared to be active in hemagglutination, hemolysis, and cell fusion assays. Levels of synthesis were in the order of 50 to 150 mg of protein per 10^8 cells.

The baculovirus-insect cell expression system has gained wide popularity as a means of expressing foreign genes for high-level production of relevant proteins (27, 36, 55). Baculovirus expression vectors use the strong, efficient promoter from the polyhedrin gene to direct transcription of the foreign gene. Polyhedrin protein is normally synthesized very late in infection (24 to 72 h postinfection) and can account for 20 to 50% of the total protein made in infected cells. This protein, which is not essential for virus replication, associates with virions in the nucleus to produce protective structures called occlusion bodies. These bodies can easily be discerned by light microscopy (59). The underlying principle behind this expression system relies upon vectors which direct recombination and substitution of the polyhedrin gene with the foreign gene of interest. Recombinant virus is produced, which forms plaques that are occlusion body negative and express the foreign gene product to various degrees.

The most widely used transfer vectors for introducing foreign genes into wild-type baculovirus are pAc373 (50, 51), pE-55 (35), p89B310 (15, 31), pAcYM1 (32), and pVL941 (29). These vectors contain the 5′- and 3′-flanking regions of the polyhedrin gene, the polyhedrin promoter and polyadenylation site, and pUC8 Amp^r for growing the plasmid in *Escherichia coli* bacterial cultures. Insertion of the recombinant gene into the wild-type virus genome relies upon a process of homologous recombination between the flanking sequences of the vector and wild-type DNA. Other vectors consisting of fused polyhedrin and foreign gene-coding sequences have also been developed (28).

Screening recombinant viruses and purifying virus containing the foreign gene away from contaminating wild-type virus can be laborious and time-consuming. This process can involve several rounds of plaque purification by using visual screening or hybridization techniques to detect recombinant plaques. An approach whereby two different promoters controlling β-galactosidase and foreign gene expression recombine as a unit with wild-type viral DNA has proven to be very successful with vaccinia virus (5). Recombinant virus was visualized as blue plaques when β-galactosidase indicator was included in the agar overlay of plaque assays. Recently Pennock et al. (40) have described a baculovirus vector which contains β-galactosidase under control of the polyhedrin promoter together with a unique *Pst*I site for

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insertion of foreign genes under their own promoter control. This vector was used to express chloramphenicol acetyltransferase controlled by the promoter from the long terminal repeat of Rous sarcoma virus (4). Low levels of chloramphenicol acetyltransferase were synthesized in lepidopteran, dipteran, and mammalian cells. Both promoters were tissue specific, and viral replication was host dependent and occurred only in lepidopteran Sf21 cells. Investigators in another laboratory constructed a baculovirus expression vector which contained two polyhedrin gene promoters (10). One promoter controlled synthesis of the normal occlusion body protein, and the other directed synthesis of the nucleocapsid protein from lymphocytic choriomeningitis virus. This vector produced occluded recombinant virus. No baculovirus vector expressing foreign genes under control of the strong polyhedrin promoter together with another promoter controlling β-galactosidase synthesis has yet been described. Such a vector would greatly facilitate the screening and purification of recombinant virus by generating blue recombinant colonies in plaque assays.

Our laboratory has been interested in obtaining large quantities of viral membrane proteins for the purpose of studying virus-host cell interactions. For this reason, we decided to express the two membrane glycoproteins of measles virus in the baculovirus expression system. The membrane fusion (F) and hemagglutinin (H) proteins of measles virus have been cloned and sequenced in our laboratories (1, 43). The H protein is responsible for host cell attachment and confers hemagglutination activity to the virus. The F protein, on the other hand, directs penetration of the host cell by the virus, causes formation of syncytia or giant cells, and mediates hemolysis of erythrocytes. All these activities involve membrane fusion and require processing of a precursor protein (F_0) by a cellular protease (47–49) to yield two disulfide-linked subunits (F_1 and F_2). By analogy, the *env* protein of human immunodefficienty virus is also cleaved by proteases and also possesses membrane fusion activity (26, 30, 33, 53).

The H protein of measles virus was recently expressed in an adenovirus helper-free vector system at levels 65 to 130% of those seen in cells infected with measles virus (2). This system produces functional H protein with accurate glycosylation and cell surface expression. Other paramyxovirus glycoproteins have been expressed in a helper-dependent simian virus 40 (SV40) vector system (38) and the vaccinia virus expression system (39, 54, 60). Levels of expression in these systems are disappointingly low, however. In an attempt to increase the production of recombinant protein, the hemagglutinin-neuraminidase protein of parainfluenza virus type 3 has been synthesized in a baculovirus expression system (7).

In this paper we report the construction of an improved baculovirus expression vector designed to accelerate the screening of recombinant virus and permit oligonucleotide-directed mutagenesis. This vector contained two promoters active very late in infection: the P10 promoter and polyhedrin promoter. P10 is a protein synthesized very late in infection, and it plays some role in the assembly of occlusion bodies (52). The sequences of the P10 promoter and P10 gene product have been reported (21, 24). The P10 promoter has been used in our laboratory to direct the synthesis of β-galactosidase while the polyhedrin promoter controlled the synthesis of the foreign gene products. The two genes, together with their promoters, recombined at high frequency with wild-type viral DNA to yield recombinant virus, which produced blue plaques when infected cells were overlaid with agarose containing β-galactosidase indicator. An origin of replication from f1 bacteriophage was included in the plasmid construction to facilitate the synthesis of single-stranded DNA (ssDNA), which could subsequently be used to introduce mutations with oligonucleotides (34, 46). This vector was used to express the F and H genes of measles virus in Sf9 insect cells. These proteins were produced in large quantities and were biologically active in hemagglutination, hemolysis, and cell fusion assays.

MATERIALS AND METHODS

Cells and Virus. *Spodoptera frugiperda* (SF9) insect cells and *Autographa californica* nuclear polyhedrosis virus (AcNPV) were obtained from the laboratory of Max Summers (Texas A & M University, College Station, Tex.). Cells were cultured in Grace medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum, TC Yeastolate (Difco Laboratories, Detroit, Mich.), lactalbumin hydrosylate, 50 μg of gentamicin sulfate per ml, and 2.5 μg of amphotericin B (Fungizone) per ml in either Falconware T flasks (Becton Dickinson Labware, Oxnard, Calif.) or spinner flasks (Bellco Glass, Inc., Vineland, N.J.) at 28°C by the procedures of Summers and Smith (55).

Escherichia coli DH5 and MC1061 were obtained from David Thomas, Biotechnology Research Institute, Montreal, Quebec, Canada. Bacterial cells were transformed by published methods (13).

Antisera. Antisera were prepared against either oligopeptides or purified proteins by published procedures (44). Rabbit polyclonal antiserum was obtained from the laboratories of Aimo Salmi, University of Alberta, Edmonton, Canada, and Tamas Varsanyi, Karolinska Institute, Stockholm, Sweden. Antiserum directed against the carboxy terminus of the membrane fusion protein of measles virus was prepared from the peptide NH_2-SRPGLKPDLTGTSK SYVRSL-COOH.

Chemicals and reagents. Restriction enzymes were purchased from New England BioLabs, Inc., Beverly, Mass. Oligonucleotides and oligopeptides were synthesized on 380A and 430A synthesizers (Applied Biosystems, Inc., Foster City, Calif.), respectively, at the Biotechnology Research Institute. Radioisotopes (^{125}I-labeled protein A [30 mCi/mg] and [α-^{32}P]CTP [3,000 Ci/mmol]) were from Amersham Canada Ltd., Oakville, Ontario, Canada. Radioactive probes for dot blot hybridizations were synthesized by using the Multiprime DNA-labeling system (Amersham Canada). Endoglycosidase H (endo H) and glycopeptidase F were obtained from Boehringer Mannheim Canada, Dorval, Quebec, Canada. Nitrocellulose paper was supplied by Schleicher & Schuell/Spectrex, Willowdale, Ontario, Canada. "Rainbow" molecular weight standards for proteins came from Amersham Canada. Powdered skim milk came from Carnation, Toronto, Ontario, Canada. African green monkey erythrocytes in Alsevier solution were purchased from IAF Biochemicals, Laval, Quebec, Canada. Bluo-Gal came from Bethesda Research Laboratories, Inc., Gaithersburg, Md. SeaPlaque agarose was obtained from FMC Corp., Marine Colloids Div., Rockland, Maine; Grace insect medium (without hemolymph) and fetal calf serum were obtained from GIBCO/BRL, Burlington, Ontario, Canada.

DNA plasmid and vector constructions. The vector pJV(NheI) was constructed by modifying the parent baculovirus transfer vector pAc373. These steps are summarized in Fig. 1. Purified genomic DNA from AcNPV was digested with *Eco*RI, and the *Eco*RI P fragment (24) was isolated.

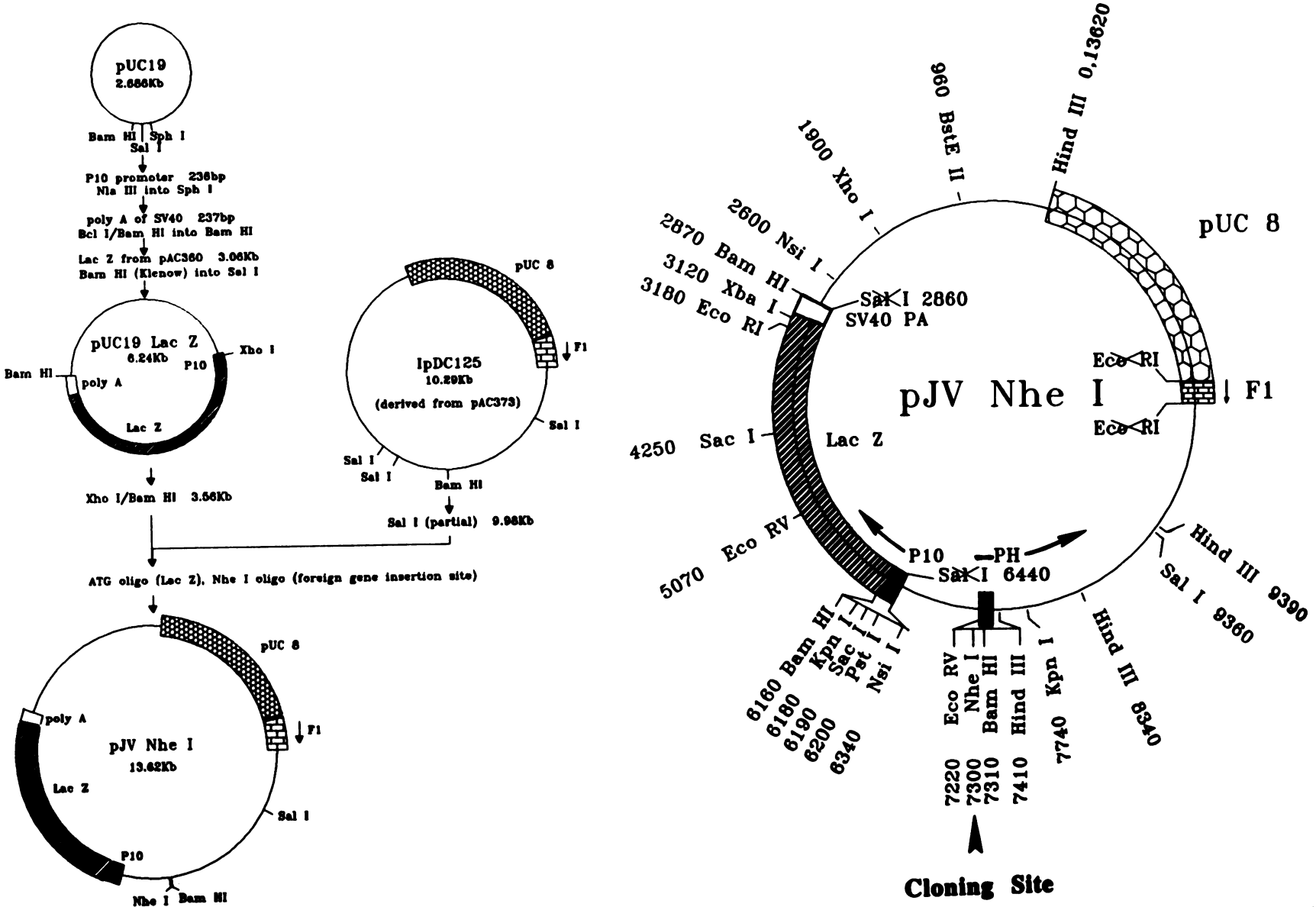


FIG. 1. Construction of the pJV(NheI) baculovirus expression vector. A transcription unit consisting of the P10 gene promoter, the β-galactosidase coding region, and an SV40 polyadenylation signal was synthesized through a series of ligations in the pUC19 shuttle vector. This modified β-galactosidase gene was inserted into the vector IpDC125 between the two *Sal*I restriction sites lying upstream of the *Bam*HI restriction site. The cloning site for foreign gene insertion was modified to contain a unique *Nhe*I restriction site and an extra 50 nucleotides from the 5′ end of the polyhedrin mRNA to increase levels of expression. Finally, an initiation codon (AUG) was added to the β-galactosidase coding region by using site-directed mutagenesis with a 63-residue oligonucleotide. A map of the restriction endonuclease sites in the vector pJV(NheI) is shown on the right. Locations of the enzymes which cleave the vector in three or fewer positions are indicated as a distance in nucleotides from the *Hin*dIII site of pUC8. *Eco*RI and *Sal*I enzyme sites which were destroyed during the ligation steps are indicated by crosses. The P10 and polyhedrin (PH) promoters, together with their direction of transcription, are indicated with arrows. Foreign genes were cloned into a unique *Nhe*I restriction site.

This DNA was digested with *Nla*III to produce a 236-nucleotide segment of DNA which contained the promoter and 5'-noncoding region of the mRNA for P10 protein. This *Nla*III fragment was inserted into the compatible *Sph*I site of pUC19. A polyadenylation signal was isolated from SV40 by using the restriction enzymes *Bcl*I and *Bam*HI and ligated into the compatible *Bam*HI site of the previous pUC19 construct. To complete the β-galactosidase transcription unit, the coding region for this enzyme was removed from the vector pAc360 (supplied by Max Summers) by using *Bam*HI. This gene was subsequently inserted into the *Sal*I restriction site of the pUC19 construct by using blunt-end ligation.

Another plasmid, IpDC125, was derived from pAc373 and was formed by including the origin of DNA replication from f1 phage in the vector. This step involved the isolation of a 514-nucleotide fragment from pEMBL8 following digestion of the DNA with *Rsa*I. The f1 origin was inserted into the *Eco*RI site of pAc373 in an orientation which yields ssDNA that is complementary to polyhedrin or foreign gene mRNA. A plasmid with the f1 origin in an opposite orientation (IpDC126) was also constructed but was not used in these experiments. IpDC125 was partially digested with *Sal*I and ligated to the *Xho*I-*Bam*HI fragment of pUC19 LacZ, which contained the transcription unit of β-galactosidase. The orientation of the various fragments was verified by using restriction endonucleases and DNA sequencing across the various junctions.

Additional changes in the modified pAc373 vector described above were made to include a unique *Nhe*I cloning site into which foreign genes could be introduced as well as to create an initiation codon for the β-galactosidase gene. ssDNA was synthesized in *E. coli* CJ236 as described below. Two oligonucleotides [(ATG oligo)ATTTACAATCATGCC TGCAGAGCTCGGTACCATGTGCAGGTCGGATCCCGT CGTTTTACAACG and (*Nhe*I oligo)CAGTTTTGTAATAA AAAAACCTATAAATATTCCGGATTATTCATACCGTC CCACCATCGGGCGTGCTAGCGGATCCTTTCCTGGGA CCCG] were annealed to this ssDNA template, elongated with T4 DNA polymerase, and introduced into competent *E. coli* DH5. Mutant plasmids were identified by mapping with restriction enzymes, and incorporation of the correct oligonucleotide was verified by DNA sequencing.

Oligonucleotide-directed in vitro mutagenesis. The site-directed modifications of the vector described above were performed by using the Muta-Gene in vitro mutagenesis kit (Bio-Rad Laboratories, Richmond, Calif.). This technique is based upon the method described by Kunkel et al. (22), in which DNA is synthesized in a *dut ung* double-mutant bacterium to produce nascent DNA which contains a number of uracils in place of thymidine. The uracil-containing strand can be used as a template for in vitro synthesis of an oligonucleotide-primed mutant strand which does not contain uracil. When the resultant double-stranded DNA (dsDNA) is transformed into normal *E. coli* strains, the uracil-containing strand is inactivated and only the non-uracil-containing strand replicates.

Blunt-end ligations. Vector DNA (5 μg) was digested with *Nhe*I, and 5' protruding ends were filled in with Klenow DNA polymerase. Vector DNA (2 μg) and insert DNA containing the foreign gene (2 μg) were ligated overnight at room temperature in the presence of 0.5 mM ATP and 2 μl (60 U) of T4 DNA ligase. Ligations were repeated the next day for 4 h after the addition of more ligase and ATP.

DNA transfections and plaque assays. Plasmids containing foreign genes were transfected into Sf9 cells together with wild-type viral DNA by using the calcium phosphate precipitation technique (55). Plaque assays were performed as previously described (55) on culture plates (100 by 15 mm). Infected cells were overlaid with 1% SeaPlaque agarose diluted with Grace medium (10 ml per plate). After a 3-day infection the culture plates were overlaid with 1% agarose in Grace medium containing 150 μg of Bluo-Gal per ml (3 ml per plate). The Bluo-Gal was dispensed from a 50-mg/ml solution in dimethylformamide. Blue spots became visible after 6 h.

Isolation of recombinant virus. Plaques which stained blue in the presence of Bluo-Gal were picked with Pasteur pipettes and placed in 1 ml of Grace medium containing 10% fetal calf serum. The virus was allowed to elute from the agarose plug overnight at room temperature. Plaque assays were again performed at 10-, 100-, and 1,000-fold dilutions. Infected cells were overlaid with agarose containing Bluo-Gal, and the blue plaques were picked after 3 days and subjected to plaque purification. Usually three to five rounds of plaque assays were sufficient to generate recombinant virus totally free from contaminating wild-type virus. Isolated recombinant virus was finally amplified in Sf9 cells to yield titers of 10^8 to 10^9 PFU/ml.

Nucleic acid dot blot hybridizations. Microdilution plates containing 24 wells were seeded with Sf9 cells at a density such that they were half confluent. The cells were then infected with either an agarose plug or 50 μl of medium containing recombinant virus and were allowed to incubate for 1 week.

Nucleic acid dot blot hybridizations were performed by a published method (55). Cells in microdilution plates were lysed with sodium hydroxide, neutralized with ammonium acetate, and spotted onto nitrocellulose paper by using a vacuum manifold. These filters were washed in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7]), air dried, and baked at 80°C for 2 h. The samples were prehybridized in 50% formamide–5× Denhardt solution–5× SSC–1% glycine–100 μg of denatured herring sperm DNA per ml for 3 h at 45°C. ^{32}P-labeled probes were prepared by using the protocol supplied with the Multiprime nucleic acid kit (Amersham Canada). Probes were prepared from 0.5 μg of purified DNA containing the foreign gene of interest. Hybridizations took place in 50% formamide–5× SSC–1× Denhardt solution–0.3% sodium dodecyl sulfate (SDS)–100 μg of denatured herring sperm DNA per ml for 12 h at 45°C. Nitrocellulose filters were then washed four times with 2× SSC–1% SDS for 10 min at 50°C and finally with 0.2× SSC–0.2% SDS for 5 min at 50°C. The filters were exposed to X-ray film overnight at −70°C.

Polyacrylamide gel electrophoresis and immunoblots. Total cellular proteins were lysed in electrophoresis sample buffer (0.06 M Tris hydrochloride [pH 6.8], 4% SDS, 40% glycerol, 3% dithiothreitol, 0.005% bromphenol blue). DNA was sheared by passage of the sample through a 26-gauge needle 10 times. Samples were applied to 8 or 10% acrylamide gels (acrylamide/bisacrylamide weight ratio, 37.5:1) and subjected to electrophoresis at 100 V overnight by the method of Laemmli (25).

Following electrophoresis, proteins were transferred to nitrocellulose sheets and probed with antibody, and antigen-antibody complexes were detected with radioiodinated protein A (3, 56). The nitrocellulose sheets were incubated for 20 min at room temperature in phosphate-buffered saline (PBS) containing 5% powdered skim milk to block nonspecific binding. The sample was then incubated for 12 h with a 1/100 dilution of antibody in PBS containing 5% milk and

0.05% sodium azide. Filters were washed once with PBS for 10 min, twice with PBS containing 0.1% Triton X-100 for 10 min, and finally again with PBS for 10 min. Radioiodinated protein A (2 μCi) was added to a 40-ml solution of 5% milk–0.05% sodium azide in PBS and incubated with shaking for 2 h. The blots were finally washed with PBS for 10 min, twice with PBS containing 1 M sodium chloride for 10 min, and finally with PBS for 10 min. These nitrocellulose sheets were finally exposed to X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) with intensifying screens for 6 to 12 h at −70°C.

Analysis of carbohydrates. Proteins were digested with glycopeptidase F or endo H by published methods (45). Cells (2×10^5) containing the appropriate recombinant protein were centrifuged at low speed, and the pellet was suspended in the appropriate enzyme incubation buffer. Incubations with glycopeptidase F were performed by using 100-μl aliquots containing 50 mM Tris hydrochloride (pH 8.6), 25 mM EDTA, 1% Triton X-100, 1% 2-mercaptoethanol, 0.2% SDS, and 0.4 U of enzyme. For endo H digestions, the samples were made up to 100 μl with 0.1 M sodium acetate (pH 5.0), 0.15 M sodium chloride, 1% Triton X-100, 1% 2-mercaptoethanol, 0.2% SDS, and 2 mU of endo H. Incubations were allowed to proceed for 8 h at 37°C. Proteins were precipitated by the addition of 250 μl of cold ethanol, briefly dried under vacuum, and subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblot analysis.

Sf9 cells (2×10^5) and Vero cells (2×10^6) were infected with recombinant baculovirus or measles virus, respectively. Tunicamycin was added to the infected cells from a 1-mg/ml stock solution in dimethyl sulfoxide. Sf9 cells were harvested at 60 h postinfection, and Vero cells were collected at 20 h postinfection. The cells were lysed in sample buffer, and proteins were subjected to electrophoresis and immunoblot analysis.

Hemolysis assays. African green monkey erythrocytes were washed twice with 50 ml of PBS and suspended in PBS to give a final 10% suspension. Incubations contained 1.2-ml aliquots of erythrocytes mixed with 200 μl of insect cells (2×10^6 cells) containing the appropriate recombinant proteins or Vero cells (2×10^6 cells) infected with measles virus. Assays were allowed to proceed at 37°C for 12 h, erythrocytes were sedimented by low-speed centrifugation, and the amount of hemoglobin released was quantitated visually or by spectrophotometric measurement at 540 nm.

Cell fusion assays. Monolayers of Sf9 cells in 24-well microdilution dishes were infected with recombinant virus containing the H and F genes at a multiplicity of infection of 3 to 5 PFU per cell. Infections were allowed to proceed for 48 to 96 h in the presence or absence of *N*-acetyltrypsin (0.5 to 2.0 μg/ml). The pH of the medium was varied by adding 0.1 M sodium citrate or PBS buffer. Syncytia formation was monitored under a phase-contrast microscope.

Hemagglutination assays. Hemagglutination titers were determined in the following manner. A 1% suspension of monkey erythrocytes was prepared in PBS. Volumes of 100 μl were aliquoted into 96-well round-bottom microdilution plates. Cells expressing H protein were serially diluted twofold in PBS and added to successive wells in 20-μl volumes. Hemagglutination was allowed to proceed overnight at 4°C.

Sf9 cells infected with H recombinant virus were incubated with monkey erythrocytes and viewed under the microscope with Nomarsky optics. Cells expressing the H protein were cultivated in 24-well microdilution dishes, and 100 μl of a 1% suspension of erythrocytes was added at various times postinfection. The erythrocytes were allowed to adsorb for 2 h at room temperature, and cells were subsequently suspended in Grace medium with a Pasteur pipette. This cell suspension was then observed under the microscope.

RESULTS

Construction of a baculovirus transfer vector containing two very late promoters and the β-galactosidase gene. A baculovirus vector was designed to incorporate features for high-level protein expression, rapid screening of recombinant virus, and effective mutagenesis of foreign genes. The pAc373 vector from the laboratory of Max Summers was modified as outlined in Fig. 1. The additions made to this plasmid are described in detail in Materials and Methods. Briefly, an f1 origin of replication from ssDNA phage was inserted into the *Eco*RI site of pAc373. This addition allows the production of ssDNA which could be packaged by MK07 or R408 helper phage. Oligonucleotides which contained desired mutations or substitutions were annealed to the ssDNA template, elongated with T4 DNA polymerase, and circularized with T4 DNA ligase. A transcription unit containing the promoter for P10 protein, the coding sequence for β-galactosidase, and the polyadenylation signal from SV40 was inserted into the polylinker region of pUC19. The assembled unit was excised and placed between the two *Sal*I restriction enzyme sites located 2,870 and 3,180 nucleotides from the *Hin*dIII site in IpDC125. Removal of this region between the two *Sal*I sites did not appear to affect the replication efficiency of recombinant virus, since titers of 10^8 to 10^9 PFU/ml were routinely obtained. Two final modifications were made by using oligonucleotide-directed mutagenesis. An initiation codon (ATG) was added to the beginning of the β-galactosidase coding region by using a 63-residue oligonucleotide (Fig. 1, ATG oligo) complementary to pAC373 at one end and the β-galactosidase gene at the other. Part of the polyhedrin-coding region and a unique *Nhe*I restriction site were inserted adjacent to the *Bam*HI restriction site of IpDC125 by using a 90-residue oligonucleotide (Fig. 1, *Nhe*I oligo). Fifty nucleotides were inserted at the *Bam*HI site, including the missing 8 nucleotides from the cap leader region of normal polyhedrin mRNA, 33 nucleotides from the coding region of the polyhedrin gene containing an initiation codon which was rendered inoperative by changing it to ATT, and a unique *Nhe*I cloning site. Such modifications purportedly increase the levels of gene expression (28, 29, 31). Oligonucleotide-directed mutagenesis and junctions between the P10 promoter, the β-galactosidase coding sequence, and the SV40 polyadenylation signal were verified by DNA sequencing techniques.

Construction of pJV(*Nhe*I) produced a vehicle which could direct homologous recombination between flanking DNA sequences of the polyhedrin gene in the wild-type virus and similar sequences in the plasmid. As a result, coding sequences for polyhedrin were replaced by a transcription unit containing two promoters which controlled β-galactosidase and foreign gene expression. A similar approach was previously shown to be very successful for the isolation of vaccinia virus recombinants (5). A restriction enzyme map of the completed vector pJV(NheI) is also presented in Fig. 1. The entire vector consisted of 13,620 nucleotides.

Coding sequences for the F and H genes were each inserted into the unique *Nhe*I cloning site of this new transfer vector. Nucleotides from the F gene coding region (1,663 bases) together with five additional nucleotides in

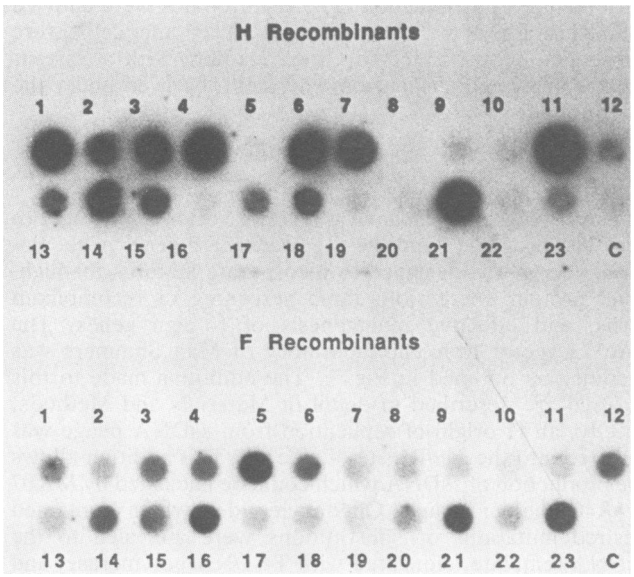


FIG. 2. Dot blot analysis of DNA produced in Sf9 cells infected with virus from the blue recombinant plaques. Agarose plugs were picked from the blue plaques and placed in microdilution wells which contained Sf9 cells. The virus was allowed to propagate for 7 to 10 days. At this time the cells were lysed and DNA was adsorbed to nitrocellulose paper. The nitrocellulose was washed, dried, and hybridized with ^{32}P-labeled probes which were specific to the H and F genes of measles virus. These hybridizations were exposed to X-ray film at −70°C for 8 h. Control cells (C) were infected with wild-type virus.

front of the initiation codon were placed in this plasmid. The H insert (1,911 bases) also contained a few extraneous nucleotides (5 bases) at its 5′ terminus along with its amino acid coding region (1,853 bases). The identity and correct orientation of these foreign genes were confirmed by partial DNA sequence analysis and restriction enzyme mapping.

Purification of recombinant baculovirus which contained the H and F genes of measles virus in addition to the β-galactosidase gene. Plasmid DNA [pJV(NheI)] which contained either the H or F gene was introduced into Sf9 insect cells together with purified viral DNA via the calcium phosphate precipitation technique. This transfection was allowed to proceed for 7 days until occlusion bodies were evident. The inoculum was diluted, and recombinant virus was purified from wild-type virus by a series of plaque titrations. At 3 days after infection, each plaque assay was further overlaid with agarose which contained a substrate for β-galactosidase (Bluo-Gal); blue plaques were usually evident after 6 h. The colored plaques continued to increase in intensity over the next day.

Approximately 0.1 to 1% of the plaques were blue, whereas wild-type virus produced white plaques containing occlusion bodies. A number of blue plaques were picked and amplified in microdilution plates which contained Sf9 cells. Lysates of these cells were spotted onto nitrocellulose membranes, hybridized with ^{32}P-labeled probes which were specific for either the F or H gene, and exposed to X-ray film. The dot blot analysis shown in Fig. 2 revealed that 15 of 23 H plaques and at least 18 of 23 F plaques contained both the β-galactosidase gene and the designated foreign gene. Control cells infected with wild-type virus did not hybridize to these probes. Negative blue plaques could be attributed either to lack of growth of virus plaques in culture or to a secondary recombination event in which the foreign gene was deleted from the recombinant.

Blue plaques contained small amounts of contaminating wild-type virus, which often pervaded during isolation of recombinant virus. However, a total of three to five rounds of plaque purification usually produced recombinant virus which did not yield occlusion bodies. The advantage of this screening system is that it permits rapid and efficient selection of recombinant plaques. Plaques produced by using recombinant virus and the pJV(NheI) vector were easier to visualize and could be detected after 3 days as opposed to 5 to 7 days when vectors which did not contain β-galactosidase were used.

Expression of recombinant H and F proteins in Sf9 insect cells. Microdilution plates containing Sf9 cells were infected with either purified recombinant virus or wild-type AcNPV at a multiplicity of infection of 5 PFU per cell. At specific times following infection, 10^5 cells were washed twice with PBS, lysed with SDS-electrophoresis sample buffer, applied to SDS-polyacrylamide gels, and subjected to electrophoresis. Proteins were transferred to nitrocellulose and probed with polyclonal antibody specific for the H or F protein. Specific binding was detected when using ^{125}I-protein A followed by autoradiography. The results of these experiments are presented in Fig. 3A and Fig. 4A. In both cases, H and F recombinant proteins appeared to be synthesized starting at 24 h postinfection and continuing up to 60 h postinfection. Infected cells appeared to be dying by 96 h postinfection.

Two species of H protein appeared to be synthesized. A high-molecular-mass species migrated at 76 kilodaltons (kDa) and comigrated with H protein made by Vero cells which were infected with measles virus. Another protein species migrated with a mobility corresponding to 65 kDa. We suspected that the 76- and 65-kDa species were glycosylated and nonglycosylated forms of the H protein, respectively. Fainter bands which migrated at low molecular masses were most probably degradation products of the H protein. The antiserum appeared to be extremely specific for the H protein, since proteins synthesized in cells infected with wild-type baculovirus failed to react with the antibodies.

Recombinant fusion (F) protein was only partially cleaved in the baculovirus expression system. A range of precursor (F_0) species (56 to 65 kDa) as well as the F_1 subunit (42 kDa) were apparent on the immunoblot autoradiogram shown in Fig. 4A. Polyclonal antisera prepared against the entire F protein failed to react with the small, carbohydrate-rich F_2 subunit (15 kDa) produced in either mammalian or insect cells. Multiple F_0 species could again be explained by differences in glycosylation by the insect cells compared with mammalian cells. The largest F_0 molecules in insect cells migrated at the same rate as F_0 in Vero monkey kidney cells. A smaller F_0 species (Fug) probably reflected incomplete glycosylation within the insect cells and appeared to be the predominant protein synthesized in cells infected with the F recombinant virus. A similar protein (Fug) was also evident in Vero cells at an early stage of infection by measles virus. Since the F_1 subunit of measles virus is not associated with sugars, it migrated exactly at the same rate as F_1 synthesized in mammalian cells. The results for F protein expression in insect cells are not surprising since these cells are known to be deficient in the terminal glycosylases and endoproteolytic enzymes involved in protein processing (19, 20, 36, 41).

Quantities of recombinant protein made in the baculovirus

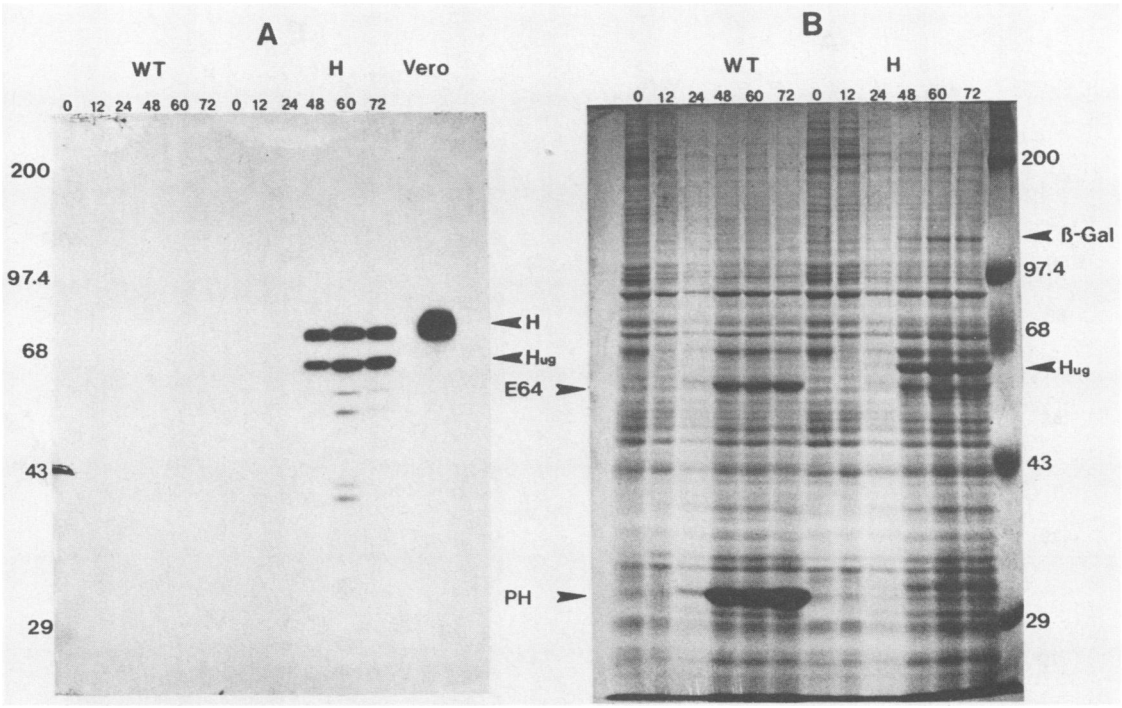


FIG. 3. Immunoblot and Coomassie blue-stained gel of total proteins produced in Sf9 cells infected with either wild-type or recombinant H virus. Sf9 cells were infected with wild-type baculovirus or recombinant virus containing the H gene of measles virus. Total proteins were solubilized by lysing the cells in sample buffer at 0, 12, 24, 48, 60, and 72 h postinfection. A positive control (lane Vero) prepared from 10^6 Vero monkey kidney cells infected with measles virus was also included. These proteins were subsequently separated by SDS-polyacrylamide gel electrophoresis. (A) Autoradiogram of an immunoblot prepared from a gel which was a duplicate of the one shown in panel B. The immunoblot was obtained by electrophoretic transfer of proteins from a polyacrylamide gel to nitrocellulose. Numbers indicate the position of colored molecular mass standards (in kilodaltons) which were transferred from the polyacrylamide gel to the nitrocellulose sheet. This nitrocellulose sheet was probed with rabbit polyclonal antiserum directed against the H protein, and antibodies bound to H protein species were detected by using ^{125}I-labeled *Staphylococcus* protein A. The immunoblot was exposed to X-ray film with an intensifying screen for 12 h at −70°C. (B) Duplicate gel stained with Coomassie blue dye. Protein standards of 200, 97.4, 68, 43, and 29 kDa are indicated by number. The polyhedrin protein (PH) is clearly evident throughout the wild-type infection but is absent in cells infected with the recombinant virus. E64 represents the major envelope protein of the baculovirus AcNPV. The electrophoretic migrations of β-galactosidase (β-Gal) and H recombinant protein products (H, Hug) are indicated with arrows. Hug refers to the unglycosylated precursor protein which migrates with a molecular mass of 65 kDa.

expression system are generally larger than those produced in mammalian systems (27). Proteins on duplicate acrylamide gels to those described above were stained with Coomassie blue and are shown in Fig. 3B and 4B. Proteins corresponding to the unglycosylated forms of H and F_0 could be detected with this dye. At least 20% of the total stained proteins could be represented by H and F polypeptide species through densitometric scanning of gel photographs. Since 250 μg of total protein (from 10^5 cells) was loaded for each lane on the electrophoretic gel, we estimated that the yields of H and F proteins synthesized in this system were in the order of 50 to 150 mg of protein per 10^8 cells. Production of H or F gene products in insect cells remained stable even after five passages of the purified recombinant virus in culture.

Glycosylation of H and F proteins in insect cells. Previous studies indicated that insect glycoproteins were first synthesized and attached to a typical high-mannose oligosaccharide, [Asn]-$GlcNAc_2$-Man_9-Glc_3 (16, 19). These mannose-rich precursors are sensitive to endo H. Further processing of N-linked glycans leads to the formation of proteins with a trimannosyl core ([Asn]-$GlcNAc_2$-Man_3) which is now endo H resistant. Since galactosyl and sialyl transferases are absent in insect cells, the trimannosyl core represents the fully processed oligosaccharide (36).

Four distinct species of F_0 precursor proteins (56 to 61 kDa) were synthesized in insect cells containing the F recombinant virus (Fig. 5). These polypeptides may represent partial glycosylation at the three asparagine sites on the F_2 subunit or may reflect various stages during the processing of the mannose-rich glycoprotein precursor (19, 43). Similar gel patterns were previously observed during immunoprecipitations of the F_0 protein from Vero monkey kidney cells infected with measles virus (43). Three species of F_0 protein were observed in these infected Vero cells. To demonstrate that the heterogeneity of F_0 was in fact due to the presence or absence of carbohydrate, insect cell lysates containing the F protein were digested with either endo H or glycopeptidase F (Fig. 5A). Endo H removes sugars from the high-mannose glycoprotein, leaving one molecule of *N*-acetylglucosamine attached to the asparagine. On the other hand, glycopeptidase F cleaves all types of asparagine-linked N-linked glycans and completely removes all carbohydrate from the glycoprotein.

The top band from the group of four F_0 polypeptides was sensitive to endo H, indicating that it represented the mannose-rich core polypeptide. The other enzyme, glycopeptidase F, completely converted the two larger species to proteins migrating with molecular masses of 56 and possibly 59 kDa. The 59-kDa polypeptide represented the major

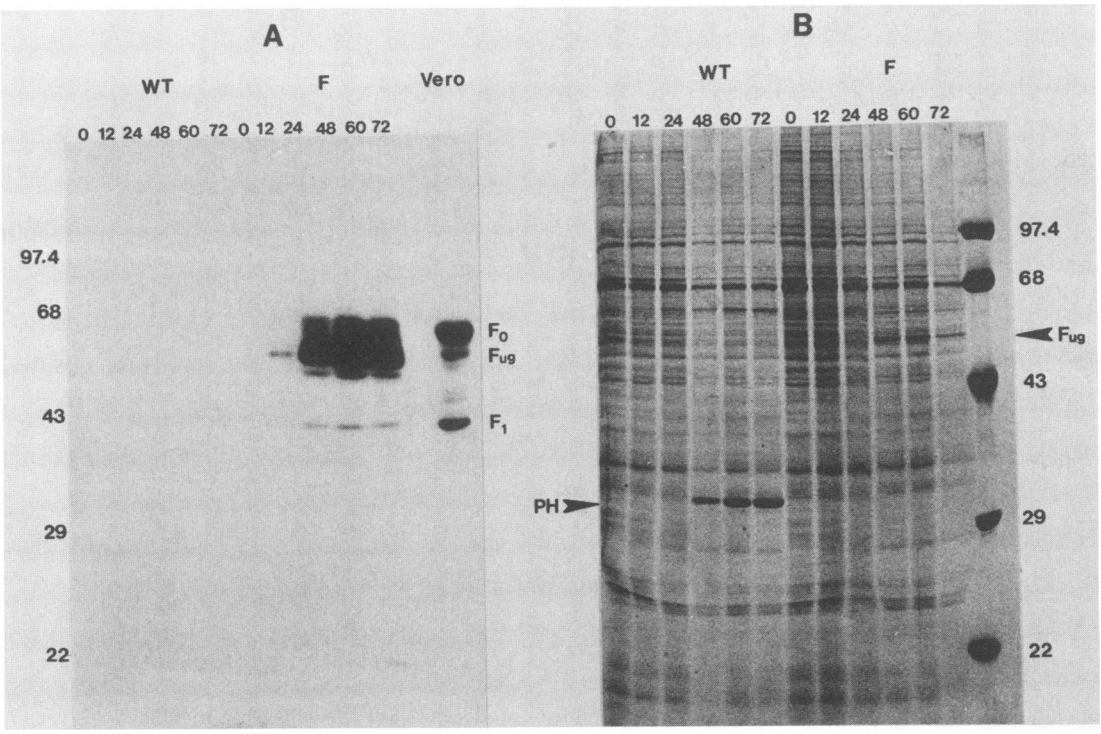


FIG. 4. Immunoblot (A) and Coomassie blue-stained gel (B) of total proteins produced in Sf9 cells infected with either wild-type or recombinant F virus. Experimental procedures were similar to those described for Fig. 3. F protein was detected on immunoblots with a polyclonal antibody directed against the entire polypeptide. The recombinant F_0 precursor protein, F_1 cleavage product, and β-galactosidase proteins are indicated by arrows. Protein molecular mass markers (in kilodaltons) are indicated by numbers. PH refers to the polyhedrin protein of AcNPV. Fug indicates the major unglycosylated species of F_0 in infected cells.

protein synthesized in Sf9 cells infected with F recombinant baculovirus. The cleaved subunit, F_1, was unaffected by glycosidases, since no carbohydrate was attached to this polypeptide (58). Unfortunately, the F_2 subunit was not detected with the polyclonal antisera made available to us.

Addition of tunicamycin (an inhibitor of Asn-linked glycosylation) to Sf9 cells infected with the F recombinant abolished the synthesis of the two largest (61 and 65 kDa) F_0 polypeptides (Fig. 5C). Two unglycosylated species (59 and 56 kDa) may correspond to the unglycosylated F_0 precursor, with and without its signal peptide at the amino terminus. Vero monkey kidney cells normally support the replication of measles virus. Tunicamycin treatment of these mammalian cells prevented the posttranslational cleavage of F_0, reduced glycosylation to yield the 56-kDa polypeptide (Fig. 5C), and also dramatically inhibited the formation of syncytia by infected cells. We hypothesized that the difference in molecular masses between the 59- and 56-kDa F_0 proteins may be due to defective processing of the membrane signal peptide by insect cells. Direct sequencing of the amino termini of these two polypeptides must be performed to substantiate this theory. Finally, further studies involving addition of radioactive sugars, pulse-chase experiments, peptide mapping, and protein sequencing are required to precisely define and identify these multiple F_0 bands.

Two major protein species (68 and 65 kDa) were recognized by antisera directed against the H protein synthesized in insect cells. To prove that the lower band represented the nonglycosylated precursor, we digested proteins with endo H and glycopeptidase F. The results are summarized in Fig. 5B. Endo H appeared to have little effect upon the higher-molecular-mass species, and this indicated that most of the mannose-rich precursor was processed to the trimannosyl core. On the other hand, glycopeptidase F completely converted the high-molecular-mass species to the smaller form. Tunicamycin treatment of infected Sf9 or Vero cells also modified the high-molecular-mass species to the faster-migrating 65-kDa protein (Fig. 5D). These experiments proved definitively that the lower protein band was a nonglycosylated form of the H polypeptide.

Recombinant H and F proteins were biologically functional in hemagglutination, hemolysis, and cell fusion assays. Recombinant H and F proteins were demonstrated to be biologically active in experiments involving cell attachment and membrane fusion assays. African green monkey erythrocytes were incubated with intact Sf9 cells which contained recombinant H protein. Hemagglutination between erythrocytes and these Sf9 cell suspensions was evident, and a reciprocal dilution titer of 2,048 was obtained. On the other hand, wild-type measles virus supernatants produced a titer of 512. Binding of erythrocytes to Sf9 cells which expressed H protein was also observed under the microscope (Fig. 6). Insect cells which contained wild-type AcNPV, or cells which were uninfected, failed to agglutinate erythrocytes. Thus, the recombinant H protein appeared to possess the cell-binding activity characteristic of the hemagglutinin molecule of measles virus.

Hemlyosis of monkey erythrocytes can be produced by measles virus. This activity can be ascribed to the cleaved membrane fusion protein of this virus. Insect cells containing H or F recombinant proteins were frozen and thawed three times and incubated with erythrocytes overnight. This treatment was previously shown to increase the hemolytic activity of measles virus (11). Hemoglobin was released into

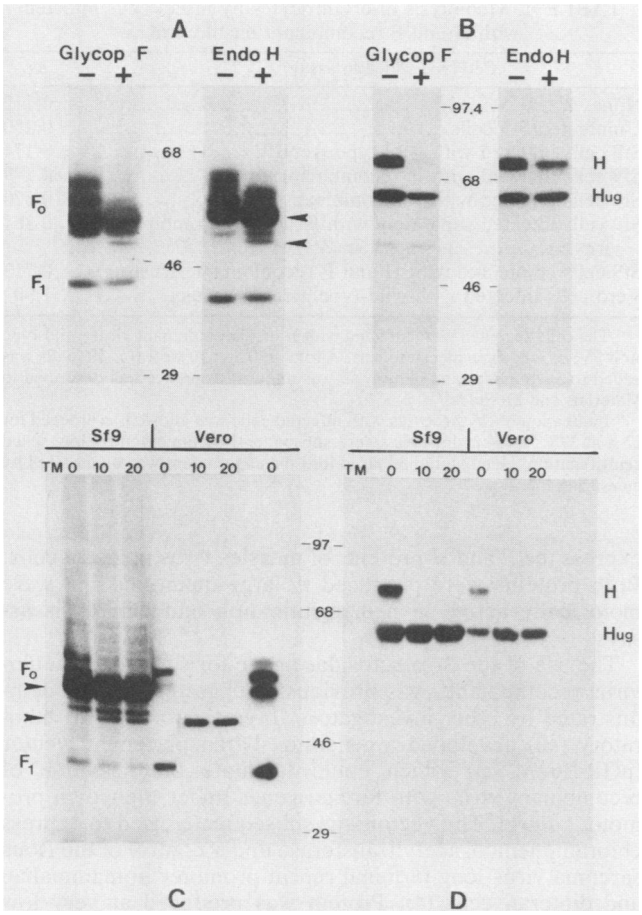


FIG. 5. Analysis of carbohydrate attached to recombinant F and H proteins by using glycopeptidase F (Glycop F), endo H, and tunicamycin (TM). Sf9 cells were infected as described for Fig. 3 and were harvested at 60 h postinfection. Vero monkey kidney cells were infected with measles virus (10 PFU per cell) and were collected at 20 h postinfection. In panels A and B, recombinant proteins from Sf9 insect cells were digested with the appropriate enzyme for 8 h as outlined in Materials and Methods. (A) Effects of endo H and glycopeptidase F digestion upon recombinant F protein; (B) effects of the enzymes upon recombinant H protein. (C and D) Effects of tunicamycin upon glycosylation of F and H proteins, respectively. Tunicamycin was present in the medium of infected Sf9 or Vero cells at concentrations of 0, 10, and 20 μg/ml. F_0 represents the precursor to the mature fusion protein, and F_1 refers to the large subunit of the active molecule. H represents the fully glycosylated hemagglutinin polypeptide, and Hug denotes the unglycosylated species. Numbers refer to the migration of colored molecular mass standards which were transferred to the nitrocellulose sheet from the polyacrylamide gel. Arrows indicate the major (59-kDa) and minor (56-kDa) unglycosylated F_0 species found in Sf9 cells infected with F recombinant virus.

the media from erythrocytes which had been treated with insect cells containing F recombinant virus or insect cells coinfected with H and F recombinant virus. H and F proteins acted synergistically to increase the level of hemolysis. This observation may reflect a requirement for cellular attachment (mediated by H) prior to membrane fusion (mediated by F) to produce an optimal level of hemolysis. Vero cells infected with wild-type measles virus also hemolyzed the erythrocytes. The level of hemolysis produced by the infected Vero cells was much greater than that elicited by insect cells infected with H and F recombinant virus. This observation may reflect a greater proportion of cleaved fusion protein at the surface of mammalian cells infected with measles virus in comparison with insect cells. Unfortunately, addition of exogenous trypsin did not increase the amount of hemolysis due to the presence of recombinant F protein. Insect cells which contained wild-type AcNPV or recombinant H protein had little effect upon the erythrocytes. These results are summarized in Table 1.

Formation of syncytia or giant cells within plaques formed by recombinant F_0 virus were not visible by phase-contrast microscopy. We attempted to demonstrate fusion of insect cells by infecting Sf9 cells with H, F, or a combination of H and F virus in the presence or absence of trypsin (0.5 to 2 μg/ml). The addition of trypsin was previously shown to increase levels of infectivity and membrane fusion activity of paramyxoviruses (14, 47). Formation of syncytia was still not evident under these conditions. The acidity of the cell culture media was then varied from a normal pH of 6.2 to 5.8, 7.0, and 8.0 since the pH requirements for membrane fusion are known to differ between groups of viruses (61). Fusion activity became dramatically apparent at pH 5.8 (Fig. 7C). Expression of F protein by itself was capable of producing polykaryons in insect cells. The addition of H recombinant virus or trypsin did not appear to enhance cell fusion substantially in this case. One might speculate that F protein expressed at the cell surface was previously cleaved en route to the plasma membrane. Also, a receptor for the viral H protein does not appear to be present on insect cells, since Sf9 cells producing recombinant H protein failed to aggregate. Thus, H protein might not be expected to enhance fusion activity between adjacent insect cells. Finally, control experiments with insect cells infected with H recombinant virus (Fig. 7A) and wild-type AcNPV (Fig. 7B) exhibited fewer syncytia and less membrane fusion activity at pH 5.8 when compared with cells infected with the F recombinant (Fig. 7C).

We concluded from these experiments that both recombinant H and F proteins were functional in biological assays for erythrocyte attachment, hemolysis, and membrane fusion.

DISCUSSION

An improved baculovirus expression vector was constructed in our laboratory, and its properties and characteristics are described in this communication. The vector was designed to accelerate the screening of recombinant virus, direct the synthesis of large quantities of protein, and facilitate oligonucleotide-directed mutagenesis of foreign genes. This DNA construct contained two promoters, from the P10 and polyhedrin genes, which are normally active very late in infections produced by AcNPV. The P10 promoter was used to direct the synthesis of β-galactosidase, an enzyme which hydrolyzes the substrate Bluo-Gal (halogenated indoyl-β-D-galactoside) to produce a dark-blue product. The other promoter, which normally regulates the synthesis of polyhedrin, was used to direct the transcription of foreign genes. An origin of replication from f1 phage was also included in the plasmid construction to facilitate the synthesis of ssDNA and subsequent mutagenesis by using complementary oligonucleotides. Both the β-galactosidase and foreign genes together with their respective promoters recombined at high frequencies with wild-type viral DNA to yield recombinant virus. This virus produced blue plaques when infected cells were overlaid with agarose containing β-galactosidase indicator. The vector pJV(NheI) was used to

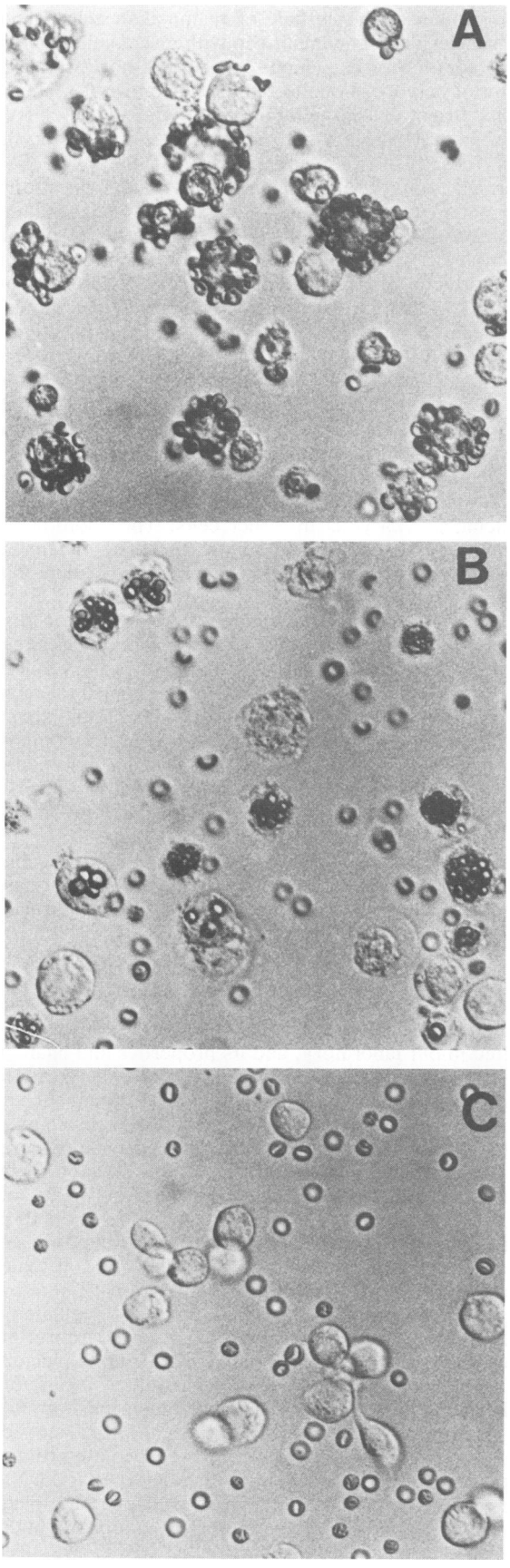


TABLE 1. Hemolysis of erythrocytes by insect cells infected with H and F recombinant baculoviruses

Cell type added to assay[a]	A_{540}[b]
None	0.112
Uninfected Sf9 cells	0.150
Sf9 cells infected with wild-type AcNPV	0.174
Sf9 cells infected with H recombinant virus	0.179
Sf9 cells infected with F recombinant virus	0.320
Sf9 cells infected separately with H and F recombinant virus	0.381
Sf9 cells coinfected with H and F recombinant viruses	0.713
Vero cells infected with wild-type measles virus	1.90

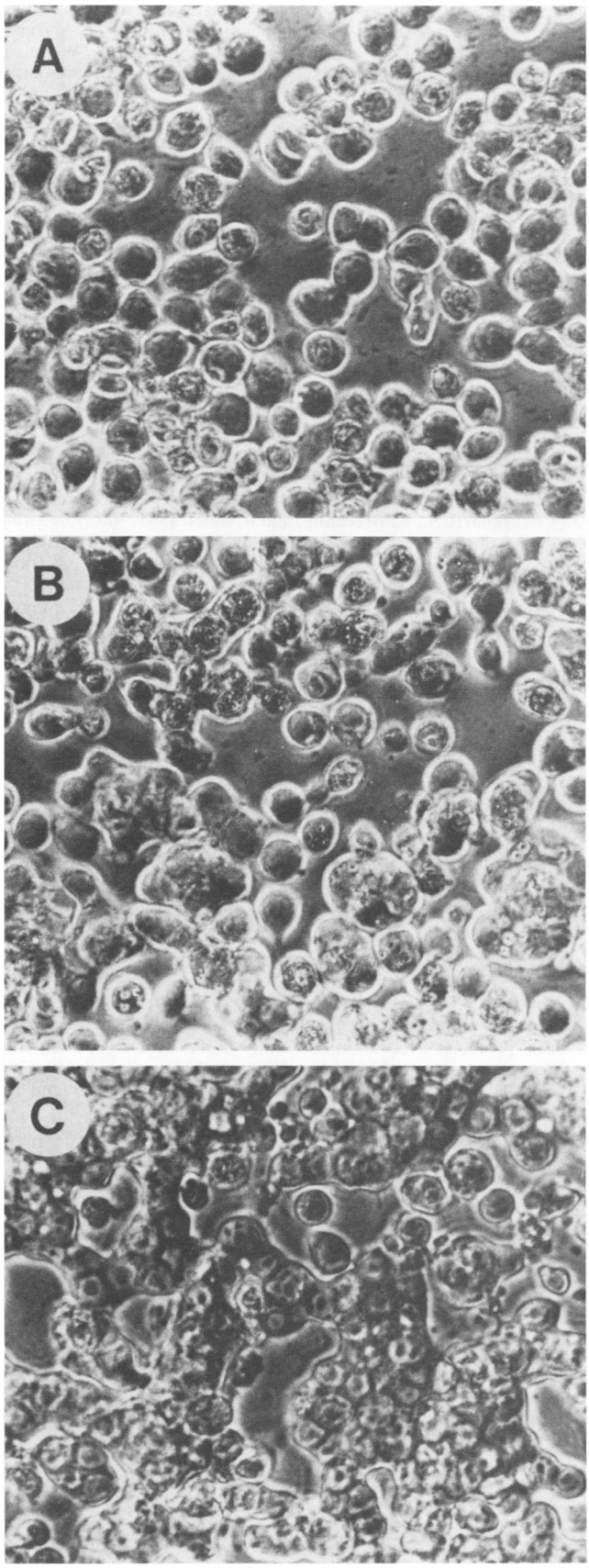
[a] The cells specified were infected with H or F recombinant virus, wild-type AcNPV, or wild-type measles virus. A total of 0.5×10^6 to 1.0×10^6 cells was added to each assay, and hemolysis was allowed to proceed as described in Materials and Methods.

[b] Incubation of erythrocytes with infected cells was allowed to proceed for 12 h at 37°C, and erythrocytes were subsequently sedimented by low-speed centrifugation. Hemoglobin released into the supernatant was quantitated by measuring the A_{540}.

express the F and H proteins of measles virus in insect cells. Both proteins were produced in large quantities and were biologically active in hemagglutination and hemolysis assays.

The use of the β-galactosidase gene for screening baculovirus recombinants was previously suggested but not demonstrated by other investigators. Investigators in this laboratory (40) developed a generalized transplacement vector (pGP-B6874/Sal) which could facilitate the selection of recombinant virus with foreign genes under their own promoter control. The vector was subsequently used to express chloramphenicol acetyltransferase under control of the Rous sarcoma virus long terminal repeat promoter in mammalian and dipteran cells (4). Protein was produced at very low levels in this experiment. Investigators in another laboratory suggested that baculovirus recombinants could be screened by the absence of β-galactosidase activity following transfection of Sf9 cells with a vector containing a foreign gene and recombinant baculovirus DNA expressing the β-galactosidase gene (55). However, this method still yields recombinant plaques which are difficult to visualize. Investigators working with vaccinia virus have previously used β-galactosidase to screen recombinant virus during expression studies (5, 57). Chakrabarti et al. (5) developed a coexpression vector (pSC11) for the isolation of vaccinia virus recombinants expressing hepatitis B surface antigen. This vector contained the hepatitis B surface antigen gene and *lacZ* gene under the control of the separate promoters P7.5 and P11, respectively. These two genes were inserted into the thymidine kinase (TK) locus of vaccinia virus and were bounded by the TK gene-flanking sequences. Tissue culture cells that had been infected with vaccinia virus were trans-

FIG. 6. Hemagglutination of monkey erythrocytes by insect cells expressing recombinant H protein. Insect cells were infected with wild-type or recombinant baculovirus for 70 h. Monkey erythrocytes were added at this time and were allowed to adsorb to infected cells for 2 h at room temperature. Cells were suspended in Grace medium and observed under the microscope with Nomarsky optics at a magnification of ×600. (A) Agglutination experiment performed with insect cells infected with the H recombinant. (B) Control experiment performed with cells infected with wild-type AcNPV. Occlusion bodies within the infected cells are evident and differ in size and density from those in adsorbed erythrocytes. (C) Uninfected cells. These failed to bind erythrocytes.



fected with the plasmid vector, and homologous recombination between wild-type virus and plasmid DNA occurred. TK^- recombinants were selected by a plaque assay on a TK^- cell line in the presence of 5-bromodeoxyuridine, which inhibited the growth of wild-type virus containing TK. Plaques that were TK^- and expressed β-galactosidase as well as hepatitis B surface antigen were shown to stain blue in the presence of indicator. The vaccinia virus expression system has the advantage of positive selection for recombinants in the presence of 5-bromodeoxyuridine. Consequently, fewer rounds of plaque purification are required with the vaccinia virus expression system than for the method developed in our laboratory for baculovirus. However, the presence of β-galactosidase in pJV(NheI) still considerably reduces the time for appearance of viral plaques.

We were concerned that insertion of the β-galactosidase transcription unit into the *Sal*I region of pAc373 might disrupt either the function of the polyhedrin promoter or the replication of the recombinant virus. However, this modification appeared to have no effect on the expression of the foreign genes under the control of the polyhedrin promoter. In fact, expression of the H gene in pJV(NheI) was consistently higher than with the pAc373 vector (C. Richardson, unpublished data). Increased levels of expression of pJV(NheI) were due to additional nucleotides inserted in the polyhedrin leader region of pAc373. Since it was difficult to purify recombinant virus from contaminating wild-type virus, we were worried that normal virus might supply some sort of helper or enhancing factor to the recombinant. However, coinfection of recombinant virus with virus-type AcNPV or another recombinant virus generated from pAc373 failed to increase levels of foreign gene or β-galactosidase expression. Recombinant virus also appeared to grow at the same rate as wild-type AcNPV once it was plaque purified. In summary, most blue plaques contained the foreign gene of interest, β-galactosidase activity, as well as the new gene product.

The criteria for optimal expression in the baculovirus system are only now being established. We patterned our expression vector after pVL941 (29), in which the initiation codon of polyhedrin protein was mutated to ATT. The nonfunctional initiation signal for translation was followed by 33 bases from the coding region of polyhedrin mRNA situated next to an *Nhe*I cloning site. The highest levels of protein expression were previously observed when portions of the coding sequence of the polyhedrin gene were fused in phase with the foreign gene (28). Other investigators have also demonstrated that 8 nucleotides adjacent to the initiation codon of polyhedrin protein are very important for efficient translation of recombinant mRNA (15, 32, 42). These modifications formed a vector which produced mRNA with a 5′ terminus similar to that of polyhedrin; this supposedly confers stability to the molecule. Future vectors will

FIG. 7. Formation of syncytia (giant cells) in Sf9 insect cells infected with the F recombinant. Monolayers of Sf9 cells were infected for 72 h with recombinant baculovirus containing the F gene. Controls were infected with either H recombinant or wild-type virus. Membrane fusion between adjacent cells was not evident at the normal pH of 6.2 found in culture medium. However, when the pH of the medium was shifted to 5.8, formation of syncytia was apparent after 2 h (panel C). Giant-cell formation was less evident in cells infected with H recombinant (panel A) and wild-type (Panel B) viruses. Cells were observed by phase-contrast microscopy at a magnification of ×600.

undoubtedly yield increased levels of expression once factors involved in transcription and translation processes within insect cells and baculoviruses are fully understood.

For the purpose of studying virus-host cell interactions, we decided to express the two membrane glycoproteins of measles virus in the baculovirus expression system. Our results indicated that both proteins were functional in hemagglutination, hemolysis, and cell fusion assays. Levels of protein expression were also impressive, since both H and F proteins could be detected on SDS-gels stained with Coomassie blue. The primary weaknesses of the insect cell expression system lie in the processes of protein glycosylation and cleavage of membrane protein precursors. Both membrane proteins were shown to be only partially glycosylated. These findings are consistent with the fact that insect cells can process the mannose-rich precursor of glycoproteins but lack galactosyl and sialic acid transferases (19, 20, 36). In most cases incomplete carbohydrate addition does not appear to impair the function of the protein. However, the role of sugars in the immunogenicity of proteins produced for vaccine development remains to be ascertained. Cleavage of the membrane fusion protein occurs in the Golgi complex of the infected cell using a host protease (37). Monkey kidney cells appear to cleave this protein efficiently, whereas Sf9 insect cells only partially process the F_0 precursor. Similar situations exist for the HA protein of influenza virus and the envelope protein of human immunodeficiency virus when they are expressed in the baculovirus system (6, 18, 23, 41). A number of mammalian cell lines were also unable to cleave the membrane fusion protein of Sendai virus (14, 47, 48). Another insect cell line, from *Trichoplusia ni*, was demonstrated to cleave F_0 more efficiently, and the activity of this product is currently being investigated in our laboratory (Richardson, unpublished).

We also noted that fusion protein synthesized in Sf9 insect cells appeared to be more active at low pH. This observation is reminiscent of the situation for membrane fusion induced by influenza virus (8, 9, 12). Influenza viruses also penetrate their host cells by membrane fusion, but this process occurs within the cells at the endosomes via a process of receptor-mediated endocytosis where the environment is acidic (pH 5.8). The difference in pH optima for fusion mediated by the fusion proteins expressed by recombinant baculovirus and measles virus was an unexpected observation (17). It is possible that the three-dimensional structure of the F protein differs in the two systems. An acid environment may be more conducive to the unfolding and exposure of the F_1 amino terminus of the recombinant baculovirus protein. It is also possible that membranes of insect and mammalian cells differ substantially in their roles as targets for fusion. These possibilities must be studied further.

Baculovirus vectors have become popular for expressing proteins of academic and industrial relevance. This publication introduces a new type of vector which facilitates faster screening of recombinant virus and efficient synthesis of foreign proteins. Newer generations of vectors which include early promoters and positive selection techniques are currently being developed in our laboratory. Finally, the gene products described in this paper are being studied to understand the early processes of viral infection and lead us toward new avenues in the treatment of viral disease.

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