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Formation of Wild-Type and Chimeric Influenza Virus-Like Particles following Simultaneous Expression of Only Four Structural Proteins

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We are studying the structural proteins and molecular interactions required for formation and release of influenza virus-like particles (VLPs) from the cell surface. To investigate these events, we generated a quadruple baculovirus recombinant that simultaneously expresses in Sf9 cells the hemagglutinin (HA), neuraminidase (NA), matrix (M1), and M2 proteins of influenza virus A/Udorn/72 (H3N2). Using this quadruple recombinant, we have been able to demonstrate by double-labeling immunofluorescence that matrix protein (M1) localizes in nuclei as well as at discrete areas of the plasma membrane where HA and NA colocalize at the cell surface. Western blot analysis of cell supernatant showed that M1, HA, and NA were secreted into the culture medium. Furthermore, these proteins comigrated in similar fractions when concentrated supernatant was subjected to differential centrifugation. Electron microscopic examination (EM) of these fractions revealed influenza VLPs bearing surface projections that closely resemble those of wild-type influenza virus. Immunogold labeling and EM demonstrated that the HA and NA were present on the surface of the VLPs. We further investigated the minimal number of structural proteins necessary for VLP assembly and release using single-gene baculovirus recombinants. Expression of M1 protein alone led to the release of vesicular particles, which in gradient centrifugation analysis migrated in a similar pattern to that of the VLPs. Immunoprecipitation of M1 protein from purified M1 vesicles, VLPs, or influenza virus showed that the relative amount of M1 protein associated with M1 vesicles or VLPs was higher than that associated with virions, suggesting that particle formation and budding is a very frequent event. Finally, the HA gene within the quadruple recombinant was replaced either by a gene encoding the G protein of vesicular stomatitis virus or by a hybrid gene containing the cytoplasmic tail and transmembrane domain of the HA and the ectodomain of the G protein. Each of these constructs was able to drive the assembly and release of VLPs, although enhanced recruitment of the G glycoprotein onto the surface of the particle was observed with the recombinant carrying a G/HA chimeric gene. The described approach to assembly of wild-type and chimeric influenza VLPs may provide a valuable tool for further investigation of viral morphogenesis and genome packaging as well as for the development of novel vaccines.

Influenza A viruses possess a segmented negative-strand RNA genome which encodes the 10 polypeptides required for effective execution of the virus life cycle. These 10 proteins are encoded within eight genomic RNA segments, each of which is encapsidated by multiple subunits of the nucleoprotein (NP) and is associated with a few molecules of the trimeric polymerase (PB1, PB2, and PA subunits) forming the functional ribonucleoprotein complex (RNP) (14).

Surrounding these structures is a layer of the matrix protein M1 that appears to serve as a nexus between the core and the viral envelope. This host-cell-derived envelope is studded with the two virally encoded major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), and a minor amount of the small nonglycosylated integral membrane protein M2 (14, 15).

Influenza virus infection is initiated by the attachment of the virus HA to a sialic acid-containing macromolecule displayed on the cell surface receptor. This virus-cell interaction initiates virus particle uptake through receptor-mediated endocytosis. The acidic endosomal environment promotes HA conformational changes that facilitate interaction of the hydrophobic NH_2 terminal domain of HA2 and the endosomal membrane. Membrane fusion results in release of the viral RNPs and matrix protein (M1) into the cytosol. Dissociation of the RNPs and matrix proteins occurs in the cytosol before the RNPs are translocated to the nucleus, where transcription and replication of the complete genome takes place (18).

Following primary transcription, newly synthesized proteins initiate replication of the viral genome, which in turn increases transcription and protein synthesis. At this point of the virus life cycle, the surface glycoproteins HA and NA start to accumulate at discrete areas of the plasma membrane from where newly assembled virus will be released. Virus assembly presumably involves interaction of cytoplasmic and/or transmembrane domains of virally encoded membrane-anchored proteins (HA, NA, and M2) and the underlying matrix protein (M1), which in turn maintains a close association with the RNPs (5, 20). The contacts between matrix protein M1 and the RNP complexes, as well as the mechanism by which a complete set of the eight required RNPs are selected and incorporated into mature virion particles, remain undefined. Specific molecular contacts amongst structural components presumably dictate the influenza virus morphogenesis pathway.

The pathway of influenza virus morphogenesis is complex and the requirements are uncertain. Numerous as yet unanswered questions include the following: (i) which viral proteins

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are required for assembly and budding? (ii) What are the protein-protein and lipid-protein interactions that drive assembly and the budding process? (iii) How do RNPs localize to a suitable assembly site and then segregate swiftly into each particle? (iv) What stoichiometric demands govern these interactions? And (v) how are assembly and budding regulated? All of these events occur in a complex cellular environment where host factors may either enhance or interfere with the progression of the assembly and budding process, leaving uncertainty concerning whether cellular proteins are indeed involved in viral assembly.

Studies of nonsegmented enveloped viruses (e.g., rhabdoviruses and paramyxoviruses) suggest that matrix protein is central to morphogenesis. Vesicular stomatitis virus (VSV) matrix protein (M) alone promotes budding of membrane particles from cell surfaces (11, 16). The M protein's singular importance also is reflected in the fact that rabies virus lacking the gene encoding the G surface protein still forms particles which are released from the infected cells (19). Similarly, human parainfluenza virus type 1 matrix and nucleocapsid proteins do associate into virus-like structures that bud from the cell surface (2). Identical rules, however, do not apply to Semliki Forest virus, where NP and M1 coexpression is inadequate for budding of membrane vesicles (29) even though M1 protein alone clearly associates with the plasma membrane (1, 13)

The recently established novel reverse genetics systems allow detailed analyses of the domains at which influenza virus-encoded proteins interact to effect budding and virion release (9, 10, 28). By this means, deletion of the influenza virus HA cytoplasmic tail decreases its incorporation into the virion envelope while compromising budding efficiency but not virion morphology. NA cytoplasmic tail deletion similarly impairs its incorporation into the viral envelope, while it enhances development of a filamentous virion morphology. Deletion of both HA and NA cytoplasmic tails seems to additively constrain budding efficiency and infectivity and to alter morphology in a way that is distinguishable from that seen with single tail deletions or wild-type virus.

Given that double HA and NA cytoplasmic tail deletions appear to affect the efficiency of budding and morphology of the virus particles without completely abrogating assembly and exit of virion particles, it seems likely that M1 protein alone may be able to direct viral assembly and budding (10). Cells expressing M1 in the absence of any other viral protein have been found to localize matrix protein to the plasma membrane (1, 13). Whether this matrix protein is completely embedded into the plasma membrane or merely attached by electrostatic interaction is uncertain. But recent work addressing this question strongly suggests that matrix-membrane association is largely, if not exclusively, the result of electrostatic interaction (24). Furthermore, amino acid substitutions in either the M1 or M2 proteins lead to changes in the morphology of the native virion, indicating that these two molecules play a key role in virion structure (22). In addition, recent studies on influenza VLP formation (7, 8) clearly demonstrate the significance of M1 protein as well as the other structural components in the assembly and budding process, which can be driven by the single expression of the M1 protein (8).

Baculovirus expression of viral proteins has been successfully used for the study of the morphogenesis of taxonomically diverse viruses (6, 12, 17, 21, 23, 25–27). Here, we have exploited recombinant baculovirus expression to define and clarify the role of specific influenza virus gene products in virion morphogenesis. A quadruple baculovirus recombinant expressing four influenza virus structural proteins (HA, NA, M1, and M2) was generated to allow definition of the molecular requirements of VLP biogenesis. Our data show that expression of four influenza virus structural proteins is sufficient for assembly and budding of VLPs from the surface of Sf9 insect cells. Expression of M1 protein alone was sufficient to promote release of vesicular particles. In contrast, neither HA, NA, nor nucleocapsid NP led to VLP formation or release. Furthermore, the full-length G protein of VSV or a hybrid HA/G was able to fully substitute for the influenza virus HA product in the process of assembly and release of chimeric particles. The implications of this study for virus morphogenesis as well as vaccine development are discussed below.

MATERIALS AND METHODS

Cloning of influenza virus HA, NA, M1, and M2 genes into the baculovirus transfer vector (pAcAB4). The influenza virus genes HA, NA, and matrix (M1) were recovered by reverse transcription-PCR (RT-PCR) from purified genomic RNA of influenza virus A/Udorn/72 (H3N2). The M2 gene, which is a spliced product of the M1 mRNA, was isolated by RT-PCR from polyadenylated mRNA extracted from MDCK cells infected with influenza virus A/Udorn/72. The two donor splice sites at the 5′ end of the M1 gene were mutated using the QuikChange kit from Stratagene (La Jolla, Calif.) (pGT-M1 splice). All the genes were cloned into pGemT or pGemT Easy vectors (Promega, Madison, Wis.) and sequenced with specific primers by using dye termination sequencing reactions and an automated ABI 377 DNA sequencer. The M2 gene was released from the pGemT-M2 plasmid by digestion with the *Eag*I restriction enzyme and prepared for cloning into the pAcAB4 (PharMingen, San Diego, Calif.) baculovirus transfer vector. The M2-DNA fragments were filled in with DNA polymerase (Klenow fragment). *Bgl*II linkers then were introduced at the termini by ligation with T4 DNA ligase. All enzymes and linkers were obtained from New England Biolabs. Thereafter, the transfer vector pAcAB4 was digested with *Bgl*II and treated with calf intestinal alkaline phosphatase (CIAP). The gel-purified M2 insert was ligated into the transfer vector at a three-to-one ratio. Desired clones were identified by restriction analysis and plasmid DNA was prepared from a 100-ml *Escherichia coli* culture using Qiagen plasmid purification kits. The resultant construction thereafter was referred to as pAcAB4/M2.

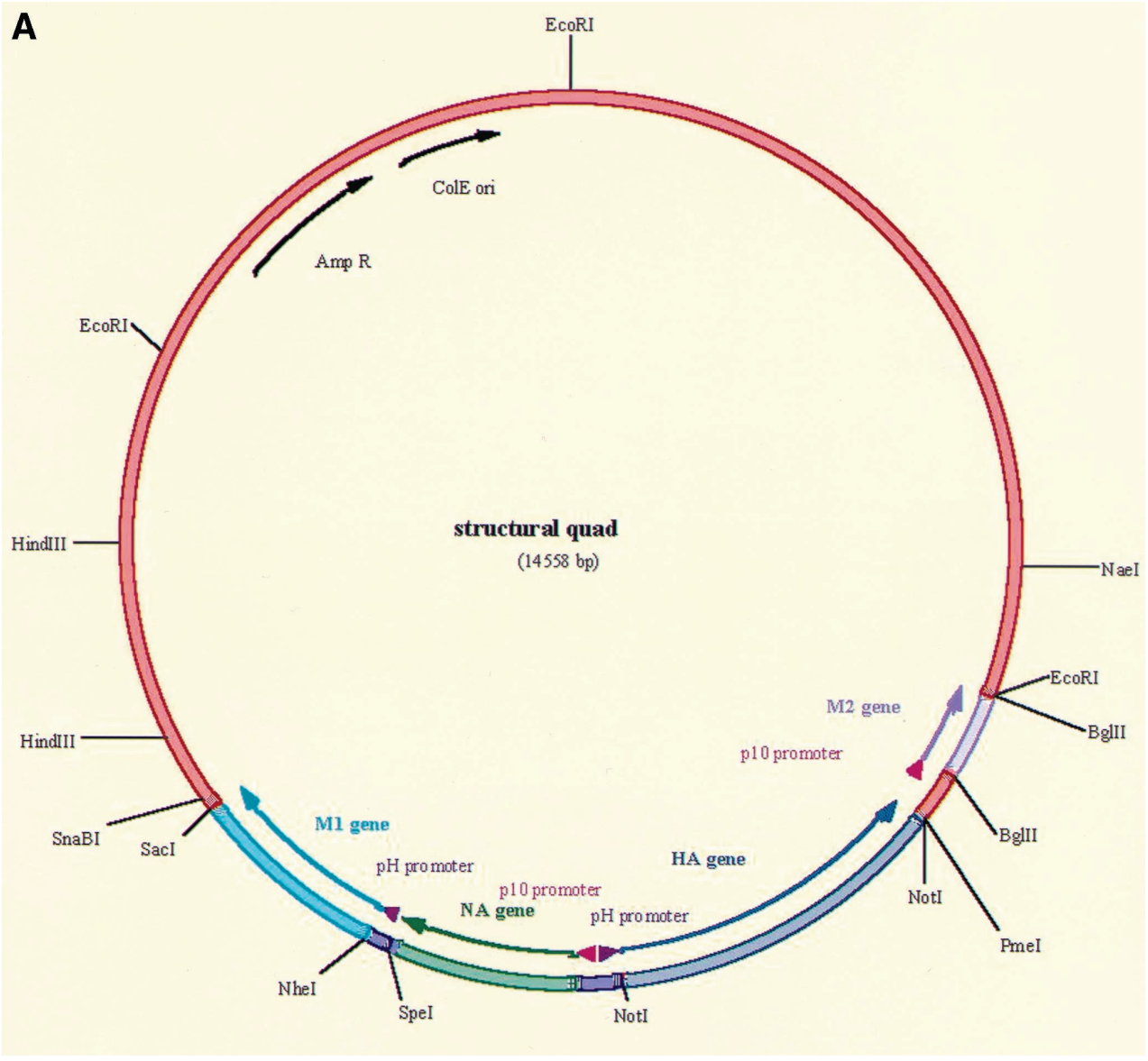
The HA, NA, and M1 genes were cloned into an intermediate vector (shuttle vector, described below) and subsequently transferred into the pAcAB4/M2. New restriction sites were introduced into the pAcAB4/M2 to facilitate cloning of DNA fragments containing these three genes from the shuttle vector.

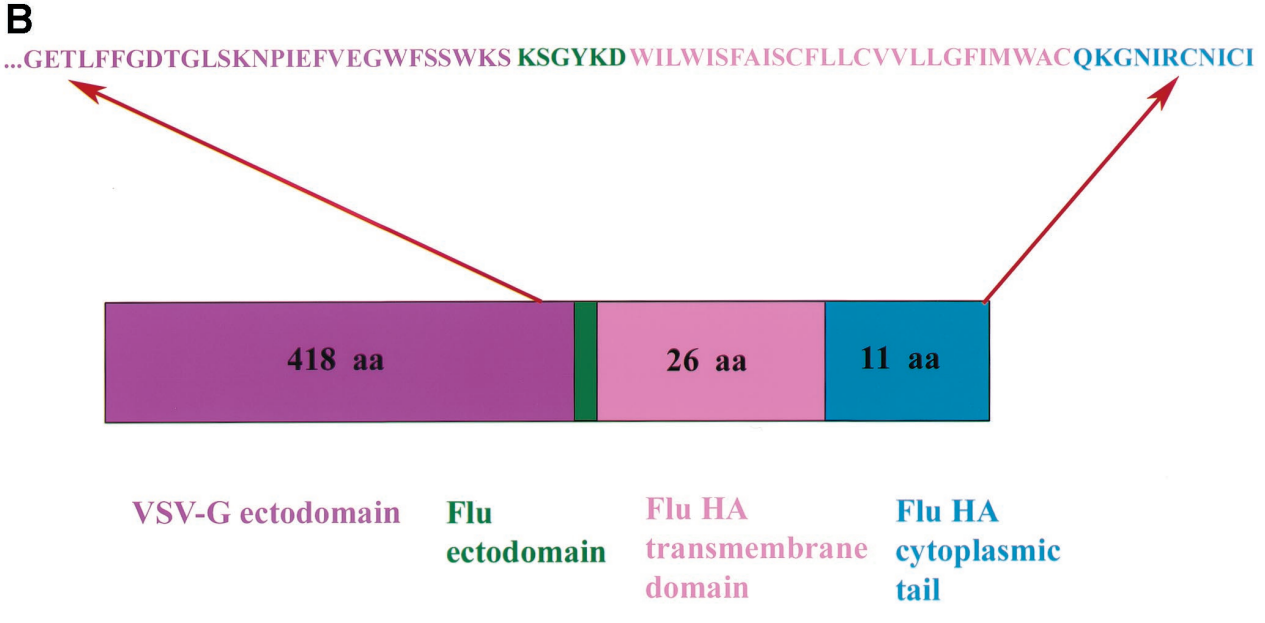
This pAcAB4/M2 plasmid was digested with *Xba*I, its termini were filled in with DNA polymerase (Klenow fragment), and *Pme*I linkers were then added with T4 DNA ligase. This DNA was then digested with *Pme*I and religated to regenerate the *Pme*I site. Similarly, the religated plasmid was digested with *Bam*HI and filled in with DNA polymerase (Klenow), and *Sac*I linkers were attached with T4 ligase. Subsequent digestion with *Sac*I and ligation restored the *Sac*I site. The resulting pAcAB4/M2 vector has two new sites, *Pme*I and *Sac*I. The vector was prepared for the insertion of the additional influenza genes by digestion with *Pme*I/*Sac*I and gel purification.

Construction of intermediate (shuttle) vector. Limited cloning sites in the transfer vector pAcAB4 prompted us to generate a shuttle cloning vector carrying three baculovirus promoters (two polyhedrin and one p10) flanked by new PCR-introduced restriction sites.

This shuttle vector was constructed as follows: pAcAB4M2 was digested sequentially with *Sma*I/*Xba*I or *Sma*I/*Bam*HI, releasing two overlapping DNA fragments, one of which contained the baculovirus promoters. In a two-step PCR, the *Pme*I/*Not*I and *Nhe*I/*Sac*I restriction sites were incorporated at each end of the PCR-rejoined fragments. This PCR DNA was then digested with *Sac*I/*Pme*I and ligated to pNEB193 plasmid (New England Biolabs), which had been linearized with the same two enzymes.

The resulting pNEB193 shuttle vector carries a DNA fragment containing two polyhedrin promoters and one p10 promoter flanked by new restriction sites suitable for the subsequent cloning of the influenza virus genes HA, NA, and M1.


A
EcoRI
ColE ori
Amp R
EcoRI
HindIII
structural quad
(14558 bp)
NaeI
EcoRI
M2 gene
BglII
p10 promoter
HindIII
BglII
SnaBI
SacI
M1 gene
pH promoter
HA gene
p10 promoter
NA gene
pH promoter
NotI
PmeI
NheI
SpeI
NotI


B
...GETLFFGDTGLSKNPIEFVEGWFSSWKS KSGYKD WILWISFAISCFLLCVVLLGFIMWAC QKGNIRCNICI
418 aa
26 aa
11 aa
VSV-G ectodomain
Flu ectodomain
Flu HA transmembrane domain
Flu HA cytoplasmic tail

These genes were cloned into the shuttle vector in three steps. (i) Cloning of the M1 gene downstream of the polyhedrin promoter was performed. The pGemT-M1 (splice) served as template in a PCR using primers specific for the gene termini that introduced at each end of the amplified gene a *Nhe*I and a *Sac*I site. The M1 gene was then subcloned into the shuttle vector using the *Nhe*I and *Sac*I sites, and its integrity was verified by BigDye (Perkin-Elmer) sequencing. (ii) Step two entailed cloning of the HA gene downstream of the polyhedrin promoter. The pGemT-HA plasmid was digested with *Sac*II, termini were filled in, and *Not*I linkers were ligated. The HA insert then was released by *Not*I digestion and introduced into the singular *Not*I site within the pNEB193-M1. Orientation of the HA gene inserted behind the polyhedrin promoter was determined by PCR and sequencing. (iii) In the third step, cloning of the NA gene downstream of the p10 promoter was performed. The pGemT-NA3B was first digested with *Sac*II and the termini were filled in with T4 DNA polymerase. Subsequently, the NA gene was released by digestion with *Spe*I and filled in with Klenow, making the NA insert blunt ended. pNEB193M1/HA was digested with *Sma*I, and the NA insert was blunt-end ligated. PCR was used to identify the clone that carried the NA gene in the correct orientation.

The shuttle vector was digested with *Pme*I/*Sac*I to release the three influenza virus genes as one piece of DNA. This DNA fragment was ligated into pAcAB4M2, which had been digested with the same enzymes (see above). All junctions were sequenced to make certain that no mutations were introduced during the pNEB193 cloning. We refer to this construct as HA/Q, which is a transfer vector carrying four influenza virus genes (pAcAB4/M2/M1/HA/NA.)

Generation of chimeric transfer vectors. The VSV-G gene was recovered from viral genomic RNA by RT-PCR using the G gene-specific 3′ and 5′ primers (5′ AACAGAGATCGATCTGT 3′ and 5′CATAAAAATTAAAAATTAAAATAT AATTAAGG 3′), and it was cloned into pGemT plasmid (Promega) for propagation. The pGemT-VSV DNA was subcloned by *Sac*II digestion and T4 DNA polymerase blunting, *Spe*I digestion, Klenow fill-in, and *Not*I linker addition. Subsequently, the *Not*I-digested VSV-G DNA was ligated into the HA/Q vector, which had been digested with *Not*I to remove its HA gene. Orientation of the gene was confirmed by PCR and sequencing. This construct was designated as the quadruple transfer vector VSV-G.

Chimeras containing the ectodomain of the VSV-G protein and the transmembrane and cytoplasmic domains of HA were constructed by PCR. The cytoplasmic tail, transmembrane domain, and 5 amino acids (aa) of the ectodomain of the influenza virus HA gene were amplified from the pGemT-HA clone by PCR using the following set of primers: 5′ **TGGAAGAGC**AAGTCAGGAT ACAAAGAC 3′ and 5′ AGTAGAAACAAGGGTGTTTTTAA 3′. The innermost HA primer (underlined) added a sequence corresponding to the VSV-G ectodomain to the PCR product (boldface). The ectodomain of the VSV-G gene was also amplified by PCR with the following set of primers: 5′ **CCTGACTT**G CTCTTCCAACTACTGA 3′ and 5′ AACAGAGATCGATCTGT 3′, where one primer incorporated a portion of HA sequence to the final PCR product (boldface). All PCR products were gel purified. These DNA fragments, which have an overlap at one of the ends, were used as template in a PCR employing *Pfu* DNA polymerase (Promega) with outside primers corresponding to the 5′ end of the VSV-G gene (5′ AACAGAGATCGATCTGT 3′) and the 3′ end of the HA gene (5′ AGTAGAAACAAGGGTGTTTTTAA 3′). A 1,620-bp DNA fragment was gel purified and *Not*I linkers were added with T4 ligase, followed by digestion with *Not*I. This insert was ligated into the quadruple transfer vector HA/Q that had been digested with *Not*I to remove the HA gene. The resultant construct generated the quadruple transfer vector VSV-G/HA (chimera).

Generation of quadruple baculovirus recombinants. Semiconfluent Sf9 cells seeded at a density of $\sim 2 \times 10^6$ onto 60-mm-diameter dishes were transfected with a mixture of approximately 2 μg of HA/Q transfer vector and 0.5 μg of linearized BaculoGold DNA (PharMingen). Recombinant baculoviruses were recovered and purified by three rounds of plaque purification. The quadruple baculovirus recombinant HA/Q28 was selected for the experiments described in this study. VSV/Q and VSV-G/HA/Q recombinant viruses were recovered similarly. All recombinants were amplified in Sf9 cells to a titer of 7×10^7 PFU/ml.

Cloning of single influenza virus genes into transfer vector pBlueBac4.5 and generation of single baculovirus recombinants. The M1 gene of influenza virus A/Udorn was previously cloned into pGemT (pGT-M1 splice, see above). To subclone the M1 gene, pGT-M1 plasmid was digested with *Sac*II and blunt ended, and terminal *Sac*I linkers were added with T4 ligase. Following plasmid digestion with *Sac*I/*Sal*I and gel purification of the released M1 gene, the M1 insert was ligated into *Sac*I/*Sal*I-digested pBlueBac4.5 and DH5α cells were transformed with the ligation mix. To subclone the HA gene into pBlueBac4.5, pGemT/HA (see above) was digested with *Sac*II and blunt ended with T4 DNA Polymerase. The DNA was then redigested with *Sal*I to remove the insert. The gel-purified HA insert was ligated into *Nhe*I (blunt)/*Sal*I-digested pBlueBac 4.5 and Stbl2 (Life Technologies, Rockville, Md.) cells were transformed with the ligation mix.

pGemT-NA (see above) was digested with *Sac*II and blunt ended with T4 DNA polymerase. This DNA was then digested with *Spe*I, NA was gel purified, and the insert was ligated into a pBlueBac4.5 vector that had been digested with *Nhe*I/*Sma*I. Stbl2 cells were transformed with the ligation mixture.

Sequence analyses were performed to verify that each transfer vector DNA carried the desired HA, NA, or M1 gene. Thereafter, Sf9 cells were transfected with 5 μg of each pBlueBac clone and 0.5 μg of Bac & Blue DNA (Invitrogen, Carlsbad, Calif.) by using a liposome-mediated process. Cells were incubated for 5 days and virus in the supernatant was subjected to plaque purification. Single blue plaques were grown and amplified in Sf9 cells, and protein expression was evaluated by Western blotting. Construction of the NP baculovirus recombinant used in this work has been previously described (4).

Growth of Sf9 insect cells and infection with baculovirus recombinants. All Sf9 cell cultures were grown in suspension in serum-free medium. Infections with baculovirus recombinants were carried out at a multiplicity of infection (MOI) of 5. Cell monolayers were infected with recombinant viruses using small inocula (1 to 2 ml), allowed to adsorb for 30 min at room temperature, and then incubated at 28°C after addition of fresh serum-free medium. Cells and culture media were collected 72 h postinfection, unless otherwise specified, and used in the subsequent analyses of protein expression and VLP formation.

Anti-influenza virus antibodies. The antibodies used in this work were obtained from the following sources: rat monoclonal anti-HA (clone 3F10), mouse monoclonal anti-HA (clone 12CA5), and mouse monoclonal anti-VSV-G (clone P5D4) were obtained from Roche Molecular Biochemicals (Indianapolis, Ind.); mouse monoclonal anti-M1 (clone GA2B) was from Serotec (Raleigh, N.C.); goat polyclonal anti-M1 was from Accurate Chemical & Scientific Corp. (Westbury, N.Y.); mouse monoclonal anti-NA (clone ST9D2) was from Argene Inc. (Massapequa, N.Y.); mouse monoclonal anti-M2 was from Mt. Sinai Hybridoma Center (New York City, N.Y.); and monoclonal anti-VSV-G (I1 and I14) and polyclonal anti-VSV-G (6794) were gifts from Jack Rose, Department of Pathology, Yale University (New Haven, Conn.).

Fluorescein isothiocyanate (FITC)-conjugated sheep anti-rabbit immunoglobulin G (IgG) IgG (whole molecule) and sheep anti-mouse IgG (whole molecule) as well as rhodamine-conjugated rabbit anti-goat IgG (whole molecule) were from Sigma (St. Louis, Mo.). Alexa Flour 594-conjugated goat anti-rat IgG (H+L) and rhodamine red X-conjugated goat anti-rabbit IgG (H+L) were from Molecular Probes (Eugene, Oreg.). AP-conjugated anti-mouse IgG (for Western blotting) was from Promega.

Immunoblot analysis. Western blot analysis was used to identify proteins in infected cells, concentrated supernatants, and iodixanol gradient fractions. Samples were resolved on a sodium dodecyl sulfate (SDS)–4 to 20% polyacrylamide gel (unless otherwise specified) and transferred onto a nitrocellulose membrane. Blots were blocked in a solution of Tris-buffered saline containing 5% nonfat dry milk and 0.1% Tween 20 and subsequently probed with a mixture of monoclonal antibodies specific for the influenza virus HA, M1, and M2 proteins. Antigens were visualized with an AP-conjugated anti-mouse secondary antibody as described above. Analysis of VLP formation in Sf9 cells infected with single-gene baculovirus recombinants was carried out in a similar fashion. Samples containing the VSV-G protein were probed with an anti-G antibody in combination with the anti-influenza virus M1 and M2 antibodies.

Immunoflourescent examination of infected Sf9 cells. Sf9 cells were grown in eight square-chamber slides and infected with quadruple- or single-gene baculovirus recombinants at an MOI of 1. At 72 h postinfection, Sf9 cells were fixed

FIG. 1. (A) Diagram of quadruple baculovirus transfer vector encoding influenza virus genes HA, NA, M1, and M2. This vector was transfected together with linearized baculovirus DNA into Sf9 insect cells to generate the quadruple recombinant (HA/Q28). Replacement of the HA with a gene encoding a full-length sequence of the VSV-G protein or chimera VSV-G/HA (see below) generated VSV-G/Q or VSV-G/HA/Q, respectively. (B) Structure of the VSV-G/HA chimera. The 11 aa of the cytoplasmic tail, 26 aa of the transmembrane domain, and 5 aa of the ectodomain of the influenza virus HA were fused in frame with the ectodomain of the VSV-G surface glycoprotein. This hybrid gene was used to replace the HA from the quadruple transfer vector (panel A) and generate the VSV-G/HA/Q gene.

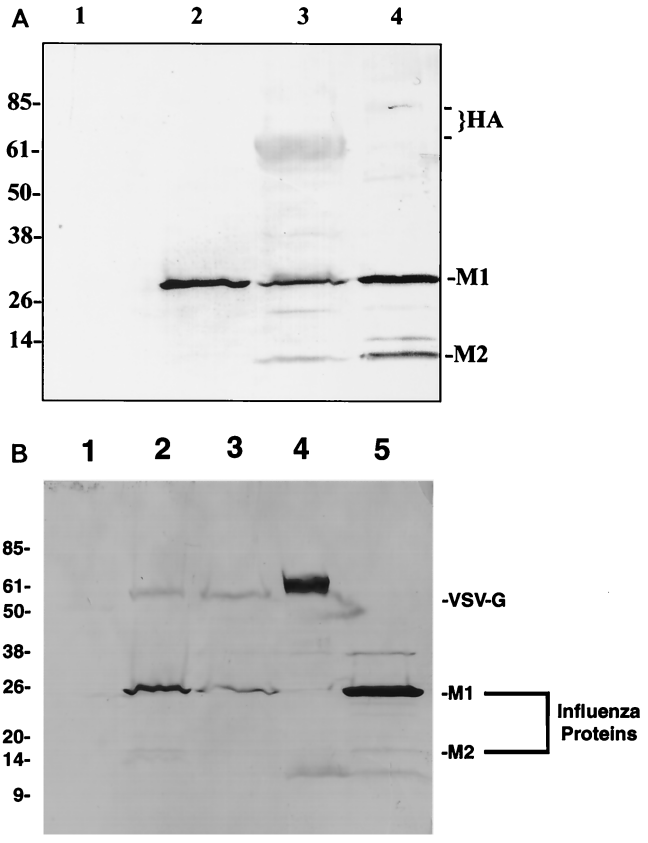


FIG. 2. Western blot analysis of the influenza virus and VSV-G proteins expressed in Sf9 cells infected by quadruple baculovirus recombinants (HA/Q28 or VSV-G/Q). (A) Expression of the influenza virus proteins HA, NA, and M2 in the cell pellet (lane 2) and culture supernatant (lane 3) was evaluated with a mixture of anti-HA, anti-M1, and anti-M2 monoclonal antibodies. Uninfected Sf9 cells and influenza virus A/Udorn-infected MDCK cells were used as controls (lanes 1 and 4, respectively). (B) Expression of VSV-G as well as influenza virus M1 and M2 proteins in Sf9 cells infected with VSV-G/Q (full-length G) was evaluated in cell pellets (lane 2) and culture supernatants (lane 3) by using a mixture of anti-G, anti-M1, and anti-M2 monoclonal antibodies. Uninfected Sf9 cells (lane 1), and VSV-infected BHK cells (lane 4) and influenza virus A/Udorn-infected MDCK cells (lane 5) were used as negative and positive controls, respectively.

in either methanol-acetone (2:1) or 3% paraformaldehyde. As a blocking step, fixed cells were incubated for 30 min at room temperature in phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA). Subsequently, each slide was incubated in a primary antibody solution of either a rat anti-HA and mouse anti-M1 (diluted 1:100 in 3% BSA-PBS) mixture or rabbit anti-NA and rat anti-HA (diluted 1:100) mixture. A combination of goat anti-rat rhodamine-conjugated antibody and sheep anti-mouse FITC-conjugated antibody or of goat anti-rat rhodamine-conjugated antibody and sheep anti-rabbit FITC-conjugated antibody was used as secondary antibodies to examine the expression of HA/M1 and HA/NA, respectively.

Evaluation of M2 expression was carried out with a rabbit anti-M2 peptide as primary antibody and a sheep anti-rabbit FITC conjugate as secondary antibody. A mouse monoclonal anti-VSV-G as primary antibody and sheep anti-mouse FITC-conjugated as secondary antibody were used to examine expression of VSV-G protein.

Each antibody reaction mixture was incubated for 30 min at room temperature, and between steps slides were rinsed three times with PBS. The FITC and rhodamine molecules emit green and red light, respectively, when excited at wavelengths of 495 and 552 nm, which were discriminated with appropriate filters.

Purification of influenza VLPs. Sf9 cells were seeded at a density of 4.5×10^7 cells per 150-cm^2 tissue culture flask and allowed to settle at room temperature for 30 min. Cells were infected with baculovirus recombinants (HA/Q28A, VSV-G/Q, VSV-G/HA/Q chimera, or single recombinant) at an MOI of 5 and infection was allowed to proceed for 72 h at 28°C. Culture medium then was harvested, subjected to low-speed centrifugation (30 min at 4°C and 2,000 × *g*), and particles were pelleted by centrifugation of the supernatant at 200,000 × *g* for 90 min. Depending on the number of cells initially infected, the resulting pellets were resuspended in 50 or 100 μl of 1× PBS, homogenized by a brief sonication, and then loaded on top of an iodixanol (Optiprep; Nycomed) gradient (density of 1.08 to 1.32 g/ml). The gradient was centrifuged at 200,000 × *g* for 3.5 h and fractions were collected by gravity from the bottom of the tube using a U-shaped microcapillary tube. To further purify the particles, fractions containing the VLPs were subjected to a second sucrose gradient centrifugation. For sucrose equilibrium gradient centrifugation, the previously selected fraction was dialyzed with PBS and layered onto a 20-to-60% (wt/wt) sucrose (in 100 mM NaCl, 10 mM Tris-HCl [pH 7.4], and 1 mM EDTA [NTE]) gradient and centrifuged for 22 h at 4°C and 150,000 × *g*. After centrifugation, 0.5-ml fractions were collected from the bottom of the tube, as described above, and analyzed by Western blotting after SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The fractions containing the VLPs were examined by electron microscopy (EM) and immunogold labeling with EM analysis.

EM. For negative staining and immunogold labeling, VLPs were concentrated from culture supernatant and purified by two consecutive density gradient centrifugations (see above). Aliquots of the samples were placed on plastic carbon-coated grids, washed gently with a few drops of distilled water, and stained with 2% phosphotungstic acid (pH 5.2). Samples for immunogold labeling of the surface antigens decorating the VLPs were prefixed in 4% paraformaldehyde for 5 min, placed on the grids, and washed with a few drops of distilled water. Subsequently, they were sequentially incubated facedown on top of 100-μl volumes of the following solutions: primary antibodies diluted in PBS-1% BSA for 30 min; PBS-1% BSA for three times, 5 min each; suspension of gold spheres coated with antibody against mouse IgG, diluted in PBS-1% BSA (1:10) for 30 min; and PBS-1% BSA for three times, 5 min each. Finally, they were washed with a few drops of distilled water, stained with 2% phosphotungstic acid, air-dried, and examined with a Zeiss electron microscope.

Immunoprecipitation. After purification on an Optiprep gradient, the VLPs were resuspended in 1 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and Complete Mini protease inhibitor cocktail). This solution was precleared to reduce background by adding 50 μl of protein A/G and rocking at 4°C overnight. To this supernatant, 10 μl of anti-influenza virus M1 antibody (Accurate Chemical) was added and the tube was incubated with rocking at 4°C for 1 h. A volume of 50 μl of protein A/G suspension was added to the sample and incubated overnight at 4°C with gentle rocking. Captured material was collected by centrifugation at 16,000 × *g* for 20 s.

The beads were resuspended in wash buffer 1 (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate) and rocked at 4°C for 20 min. This step was done twice. Complexes were collected by centrifugation at 16,000 × *g* for 20 s. Beads were resuspended in wash buffer 2 (50 mM Tris-HCl [pH 7.5], 0.5 M NaCl, 0.1% NP-40, 0.05% sodium deoxycholate) and rocked at 4°C for 20 min. This step was repeated once. Complexes were collected by centrifugation at 16,000 × *g* for 20 s. Beads were resuspended in wash buffer 3 (50 mM Tris-HCl [pH 7.5], 0.1% NP-40, 0.05% deoxycholate) and rocked at 4°C for 20 min. All traces of the final wash were removed from the walls and lid of the tube. The agarose pellet was resuspended in 50 μl of Laemmli buffer and denatured at 98°C for 3 min. Samples were then subjected to PAGE in 4 to 20% Tris-glycine-SDS gel, and the influenza virus protein(s) was detected by Western blot analysis.

RESULTS

Expression of influenza virus structural proteins in Sf9 insect cells infected with a quadruple (HA, NA, M1, and M2) baculovirus recombinant. To investigate the process of influenza virus assembly and budding, we constructed a series of single-gene and quadruple-gene baculovirus recombinants. Four influenza virus genes, for HA, NA, M1, and M2, were introduced into a single baculovirus recombinant for simultaneous expression of these structural proteins (Fig. 1A). Based on the level of protein expression as assessed by immunoblot-

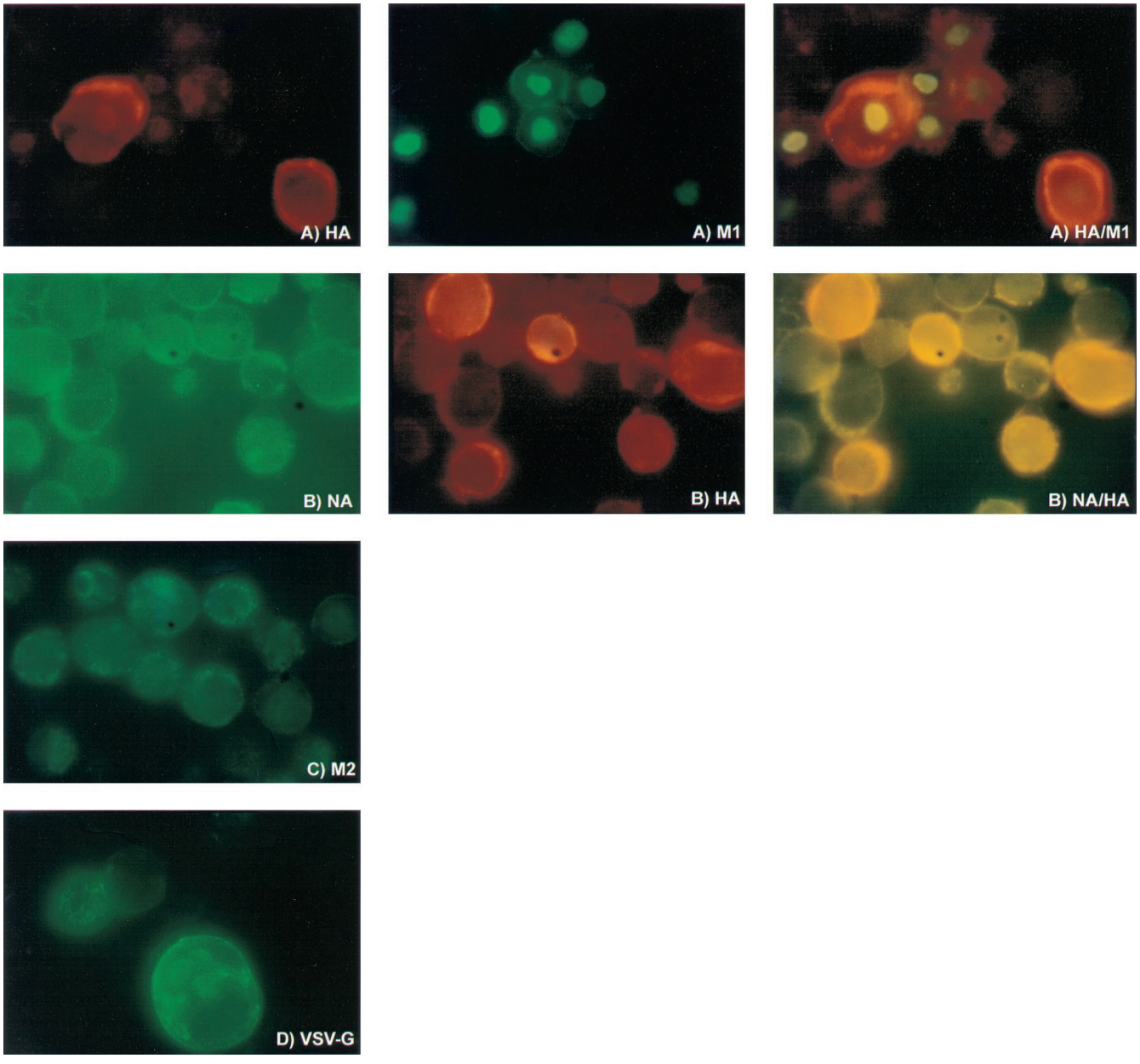
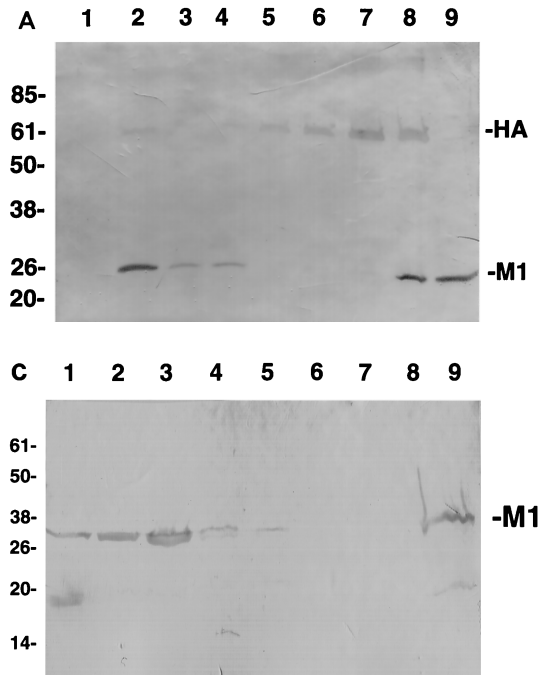


FIG. 3. Immunofluorescence analysis of Sf9 cells infected with a quadruple baculovirus recombinant (HA/Q28). Expression of the influenza virus proteins HA, M1, or HA/M1 (A) and HA, NA, or HA/NA (B) was detected with a combination of anti-HA and anti-M1 or anti-HA and anti-NA antibodies as described in Materials and Methods. (C) M2 expression was examined with a rabbit anti-M2 peptide antibody. (D) Sf9 cells infected with VSV/Q chimeric baculovirus recombinant were used to evaluate expression of the VSV-G protein.

ting, we selected the quadruple recombinant (HA/Q28) to use in subsequent experiments.

Western blot analysis of Sf9 insect cells infected with the quadruple baculovirus (HA/Q28) and probed with anti-HA and anti-M1 monoclonal antibodies demonstrated high levels of expression of the influenza virus HA, M1, and M2 proteins (Fig. 2A). Furthermore, all these proteins were secreted into the culture supernatant of the Sf9-infected cells (Fig. 2A, lane 3). The HA protein electrophoretic migration pattern differed from that of the native HA synthesized during influenza virus A/Udorn infection of MDCK cells, presumably reflecting the glycosylation limitations typical of insect cells. M1 and M2 proteins, in contradistinction, displayed electrophoretic mobilities indistinguishable from those expressed by influenza virus-infected MDCK cells (Fig. 2A, lanes 3 and 4). Expression of the NA protein was detected by Western blotting with a mouse polyclonal antibody that also recognized HA, M1, and M2 (data not shown).

To further evaluate expression and cellular localization of the influenza virus proteins in Sf9 cells, we carried out immunofluorescence analyses of cells infected with HA/Q28. These experiments demonstrated that all four influenza virus structural proteins were expressed (Fig. 3). Dual-staining experiments with anti-HA and anti-NA antibodies demonstrated that



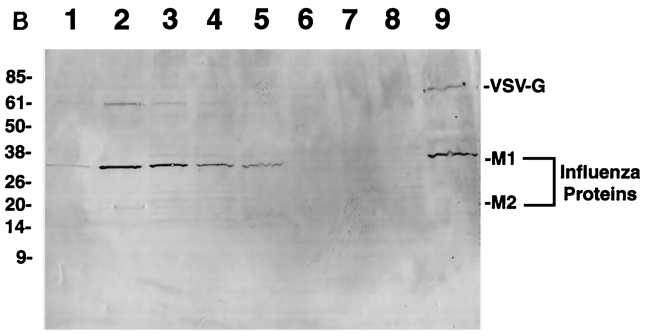


FIG. 4. Analysis of VLP formation by iodixanol gradient centrifugation. Concentrated supernatants of HA/Q28- or VSV-G/Q recombinant-infected Sf9 cells were layered on top of an iodixanol density gradient and spun at 200,000 × *g* for 3.5 h. Fractions were collected from the bottom of the tube, resolved by SDS-PAGE, and transferred onto nitrocellulose. (A) Fractions from HA/Q28 were probed with a mixture of anti-HA and anti-M1 monoclonal antibodies. Lanes 1 to 8, fractions collected from top to bottom of the tube; lane 9, influenza virus A/Udorn-infected MDCK cells as control. (B) Fractions from VSV-G/Q were probed with a mixture of anti-VSV-G, anti-M1, and anti-M2 monoclonal antibodies. Lanes 1 to 8, fractions collected from the top to the bottom of the tube; lane 9, VSV-infected BHK cells and influenza virus-infected MDCK cells combined as control. (C) Concentrated supernatants of Sf9 cells infected with the M1 single baculovirus recombinant were purified as above and probed with anti-M1 antibody. Lanes 1 to 8, gradient fractions collected from the top to the bottom of the tube; lane 9, influenza virus-infected MDCK cells (control).

these surface glycoproteins were present at the periphery of the infected cells with a degree of overlap (Fig. 3B), suggesting colocalization at discrete areas on the plasma membrane similar to that seen in natural influenza virus infection. Double immunofluorescent staining of HA/Q28-infected Sf9 cells with a mixture of monoclonal antibodies to either HA or M1 also showed cell membrane colocalization as well as accumulation of a portion of M1 in the nuclei of the infected cells (Fig. 3A). Furthermore, expression of M1 alone showed membrane and nuclear localization (data not shown), suggesting that coexpression with HA, NA, and M2 does not appear to change its cellular distribution (Fig. 3A). Simultaneous expression of M1 and NP did not show redistribution of these proteins in the cells compared to that in Sf9 cells infected individually with either M1 or NP recombinants (data not shown). Expression of the M2 protein was also detected when HA/Q28-infected Sf9 cells were evaluated by immunofluorescence. These analyses showed that M2 proteins clearly accumulated at the periphery of the infected cells with areas of differential flourescence intensities, indicating patchy localization of M2 at the surface of the cells (Fig. 3C)

Study of assembly and release of influenza VLPs from Sf9 cells infected with an HA, NA, M1, and M2 quadruple baculovirus recombinant. Given that some of these influenza virus proteins colocalized at the cell surface, we asked whether these four viral proteins were sufficient to drive the assembly and release of VLPs from the surface of the infected cell. HA/Q28-infected Sf9 cell supernatants were collected 72 h postinfection, clarified by low-speed centrifugation (2,000 × *g* for 30 min), and concentrated at 200,000 × *g* for 90 min. Western blot analysis of the pelleted material using a combination of anti-HA, -M1, and -M2 monoclonal antibodies revealed all three influenza virus proteins (Fig. 2A, lane 3). In order to investigate whether these proteins were released from the cell as a consequence of cellular damage and death or because they assembled as a VLP that budded from the cell surface, concentrated supernatants from HA/Q28-infected Sf9 cells were subjected to iodixanol (3) velocity gradient centrifugation (200,000 × *g* for 3.5 h).

Western blot analysis of collected gradient fractions showed cosedimentation of HA and M1 with a peak concentration in fraction 2 (Fig. 4A, lane 2). These proteins were also found in adjacent fractions 3 and 4 as well as in fractions 7 and 8 (Fig. 4A). Detection of HA and M1 in these lower fractions (toward the bottom of the gradient) is likely due to the association of these proteins with the baculovirus, which under these experimental conditions bands in fractions 7 and 8 (data not shown). To further characterize the nature of the association among the influenza virus proteins that comigrated into fractions 2 and 3, we carried out EM evaluation of the material present in these fractions.

Evaluation of influenza virus VLPs by EM. High concentrations of both vesicular and nonvesicular particles studded with surface projections resembling influenza virus and subviral particles were noted by EM examination of fractions 2 and 3. In order to improve the purity of the putative VLPs, fractions 2 and 3 were subjected to isopycnic centrifugation in sucrose gradients. EM analysis of this sample clearly showed the presence of VLPs that resemble the influenza virus not only in their morphological appearance but also in their characteristic surface projections (Fig. 5). The surface projections decorating some particles were very similar to the HA spikes of influenza virus. The putative NA structures were less distinctive and more difficult to identify on the surface of spike-studded entities. Fraction 3 contained a similar range of particles in high

concentration; however, it also seemed to contain a higher concentration of aggregated membranes than fraction 2 (data not shown).

We investigated the protein composition of the spikes that protruded from the surface of the particles by EM of immunogold-labeled surface antigens recognized by specific monoclonal antibodies to HA and NA. This examination showed that the major influenza virus surface antigen HA was indeed present on the surface of the VLPs (Fig. 6A), confirming what was assumed from the structural evaluation of the spikes visualized by negative-staining EM (Fig. 5). Similarly, immunogold labeling with anti-NA antibody and EM analysis also revealed the presence of NA glycoprotein on the surface of the VLPs, although at lower abundance than HA (Fig. 6B). Efforts to detect M2 protein on the surface of the VLPs by immunogold labeling using a rabbit polyclonal antibody raised against peptides encompassing 18 aa of the NH_2 terminal of the M2 protein failed even though M2 was detected by Western blotting in the same gradient fractions subjected to immunogold labeling and electron microscopy.

Assembly of chimeric influenza VLPs bearing the VSV-G protein. In order to evaluate whether heterologous glycoproteins get incorporated into the surface of the influenza VLPs, we constructed two new quadruple recombinants. In one recombinant, the HA gene was replaced by a full-length G gene of VSV, generating VSV-G/Q (Fig. 1, and see Materials and Methods). In the second recombinant, the HA gene was replaced by a VSV-G/HA chimera generating a quadruple baculovirus recombinant (G/HA-Q). This viral recombinant carried a hybrid gene encoding the ectodomain of the VSV-G protein and the transmembrane domain and cytoplasmic tail of the influenza virus HA, in addition to the three structural influenza virus genes M1, NA, and M2 (Fig. 1A and B). Immunofluorescence analysis of VSV-G/Q-infected Sf9 cells showed expression of G protein, which appeared to localize to the surface of the infected cells (Fig. 3D). Furthermore, Western blot analysis of VSV-G/Q-infected Sf9 cells showed that the VSV-G protein as well as influenza virus proteins M1 and M2 were expressed 72 h postinfection (Fig. 2B, lane 2). In addition, Western blot analysis of the concentrated media supernatants from infected cell cultures showed the presence of VSV-G and influenza virus M1 and M2 (Fig. 2B, lane 3). M2 protein was not as readily detected as it was in the supernatant of HA/Q28-infected cells (Fig. 2A, lane 3). To determine if there was an association among the proteins secreted into the culture media of infected cells, concentrated supernatants were subjected to a velocity centrifugation on an iodixanol gradient. As observed with the HA/Q28 recombinant, fraction 2 of an iodixanol gradient contained the VSV-G and the influenza virus M1 and M2 proteins (Fig. 4B, lane 2). When Sf9 cells were infected with a quadruple recombinant carrying VSV-G/HA instead of HA, all the probed proteins (VSV-G, M1, and M2) were found in both the concentrated supernatant and fractions 2 and 3 of the iodixanol gradient (data not shown). These results suggested that infection of Sf9 cells with either quadruple recombinant VSV-G/HA/Q (chimera) or VSV-G/Q (full length) directed the assembly and release of influenza VLPs bearing the VSV-G proteins on their surface. EM evaluation of the VSV-G/HAQ chimera and VSV-G/Q revealed the presence of particles that were morphologically similar to the ones released by HA/Q28, and immunodecoration with anti-VSV-G demonstrated the presence of surface-localized G protein as well (data not shown).

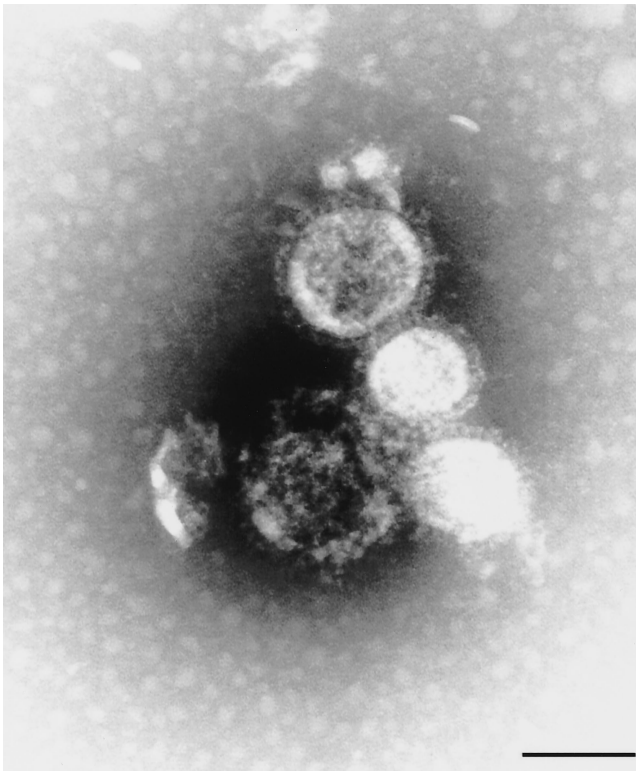


FIG. 5. Electron micrograph of negatively stained influenza VLPs gradient-purified from culture media of Sf9 cells infected with a quadruple recombinant HA/Q28. Bar, 100 nm.

Evaluation of VLP formation following Sf9 infection with one single baculovirus recombinant (M1, HA, NA, and NP) or dual infection. In view of the fact that some of the HA/Q28 particles examined by EM did not show detectable surface spikes, the question arose as to whether the influenza virus matrix protein by itself is sufficient to drive assembly and release of vesicular particles. To address this question, we infected Sf9 insect cells with an M1 baculovirus recombinant for 72 h and subjected concentrated culture supernatants to the same analysis as described above. Immunoblot analysis of iodixanol gradient fractions demonstrated that matrix protein concentrated in fractions 2 and 3, similar to the sedimentation pattern of the VLP released from Sf9 cells infected with HA/Q28 (Fig. 4C). To further characterize the structure of the M1 particles, we examined these two fractions of the gradient by EM. Negative-stained EM examination showed a large number of vesicles of various shapes that did not bear spikes on their surfaces (data not shown).

To find out whether expression of M1 protein was driving particle formation or baculovirus infection was inducing the release of membrane vesicles, we examined by Western gradient the fractions of concentrated supernatants of Sf9 cells infected with single recombinants that express the surface proteins HA or NA. We were unable to detect significant amounts of these proteins in the gradient fractions (data not shown),

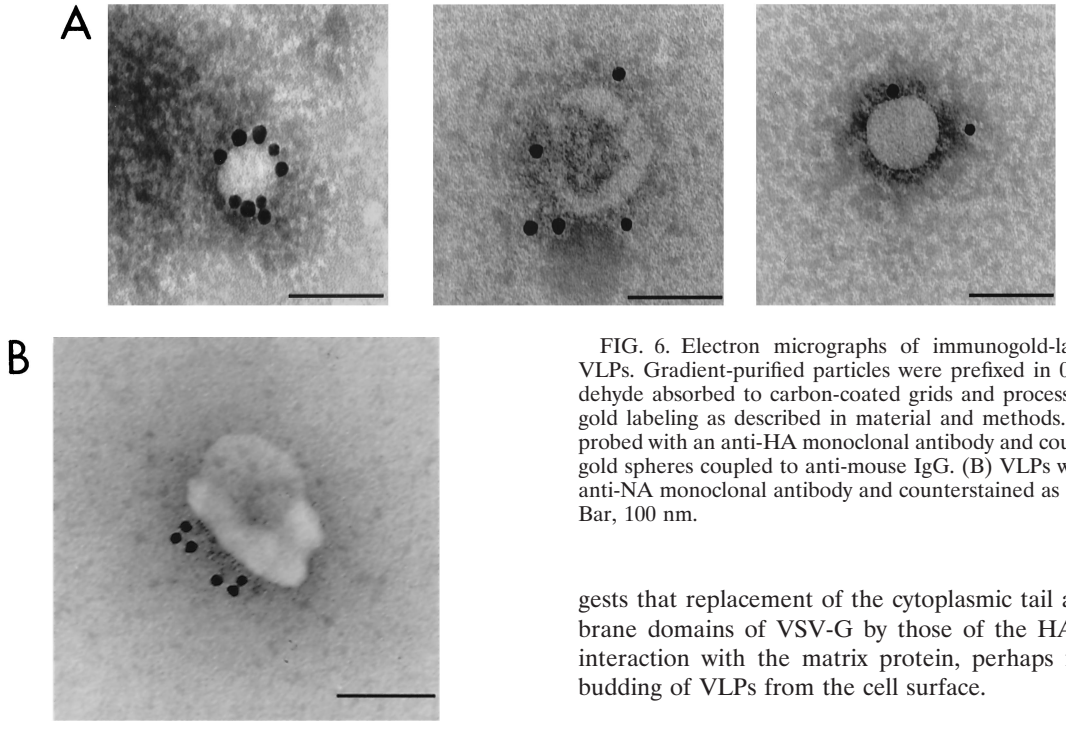


FIG. 6. Electron micrographs of immunogold-labeled influenza VLPs. Gradient-purified particles were prefixed in 0.5% of glutaraldehyde absorbed to carbon-coated grids and processed for immunogold labeling as described in material and methods. (A) VLPs were probed with an anti-HA monoclonal antibody and counterstained with gold spheres coupled to anti-mouse IgG. (B) VLPs were probed with anti-NA monoclonal antibody and counterstained as described above. Bar, 100 nm.

suggesting that the baculovirus did not promote release of membrane vesicles containing NA or HA proteins.

In order to get an insight into the frequency of VLP formation, we evaluated the relative amount of M1 protein associated with gradient-purified particles, which we assumed to be a reflection of VLP formation and release. Equal numbers of Sf9 cells were infected with HA/Q28 or M1 single recombinants and an equivalent number of MDCK cells was infected with influenza virus A/Udorn. VLPs or influenza virions released into the culture supernatant were concentrated and purified by gradient centrifugation. The M1 protein present in fractions 2 and 3 of the VLPs and fractions 3 and 4 of the virus gradients was immunoprecipitated and subsequently evaluated by Western blot analysis. We used image densitometric analysis to compare the relative amounts of M1 protein immunoprecipitated from purified influenza virus, HA/Q 28, and M1 particles (Fig. 7A). The relative amount of M1 protein obtained from HA/Q28 and the M1 single recombinant was 2 times and 1.1 times, respectively, that obtained from influenza virus (Fig. 7A, lanes 1, 2, and 3). Although this approach did not allow us to determine the VLP yield, it did suggest that VLP formation and release is not a rare event and that it is, at least in part, mediated by the M1 protein.

Similarly, the relative amounts of M1 protein and VSV-G or VSV-G/HA chimera released by Sf9 cells infected with the quadruple recombinant VSV-G/Q or VSV-G/HA/Q were determined by Western blot analysis of concentrated infected cell media supernatants. These analyses demonstrated that the relative amounts of M1 and VSV-G/HA released from Sf9 cells infected with the VSV-G/HA/Q chimera recombinant were 1.4 and 2 times higher, respectively, than that released by Sf9 cells infected with the VSV-G/Q (Fig. 7B). The increase in relative abundance of M1 and VSV-G/HA chimera glycoprotein suggests that replacement of the cytoplasmic tail and transmembrane domains of VSV-G by those of the HA enhanced its interaction with the matrix protein, perhaps facilitating the budding of VLPs from the cell surface.

DISCUSSION

In this report we described the formation of influenza VLPs directed solely by four viral structural proteins (HA, NA, M1, and M2). These VLPs are assembled and released from Sf9 insect cells coexpressing the four influenza virus proteins as directed by a quadruple baculovirus recombinant. The VLPs closely resemble influenza virus in size, particle morphology, and fine structure of the surface spikes. Furthermore, these VLPs form in the absence of influenza virus RNPs, indicating that assembly and release of particles, at least in this particular system, does not require viral RNPs. However, we cannot rule out the possibility that some cellular structure may be enhancing the budding of the VLPs. These results are in complete agreement with other recent work (8) in which VLP formation was obtained following expression of a similar set of structural proteins.

Our studies present clear evidence of the expression and appropriate localization of these four structural proteins as well as of their association and release from the cell surface as VLPs. These structures bear surface projections that stud the membrane of the particle and project outward as HA and NA do on the surface of influenza virions. Immunogold labeling and EM examination clearly identified HA protein decorating the surface of the VLPs. Similar studies also demonstrated the presence of NA protein on the surface of the VLPs, although at a much lower frequency than that observed for the HA protein. The ratio of HA to NA in influenza virions is approximately 5 to 1; therefore, this result may be a reflection of the different rate by which these two glycoproteins are incorporated onto the surface of the particle. We were not able to identify the M2 protein by immunogold labeling and EM, perhaps due to low abundance of M2 protein on the VLP surface or inaccessibility of the M2 epitopes, or simply because it was not incorporated into the particle.

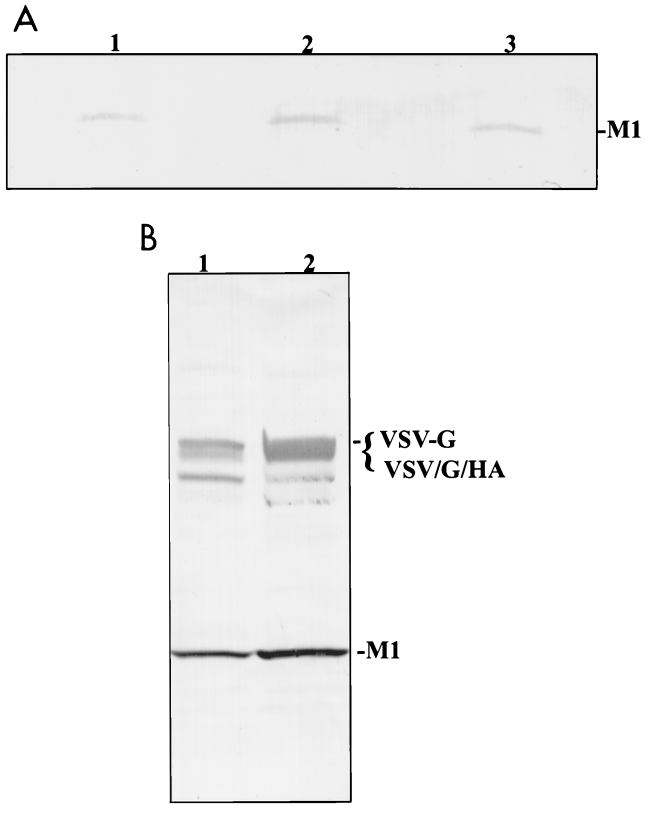


FIG. 7. (A) Comparative Western blot of M1 protein released from influenza virus A/Udorn-infected MDCK cells or from Sf9 cells infected with the quadruple recombinant HA/Q28 or the M1 single recombinant. MDCK cells (5×10^6) were infected with influenza virus A/Udorn (MOI, 1) for 24 h. Equivalent numbers of Sf9 cells were infected with either HA/Q28 or M1 single recombinant (MOI, 5) for 72 h. Supernatants were concentrated and particles were purified by iodixanol gradient centrifugation. Fractions 2 and 3 were pooled, and M1 protein was immunoprecipitated with anti-M1 monoclonal antibody twice to ensure complete recovery. Samples were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-M1 polyclonal antibody. Different amounts of each sample were analyzed to determine the linear range of the detecting color reaction. Lanes 1, 2, and 3 show the relative amount of M1 protein immunoprecipitated and detected by Western blotting from purified influenza virions, HA/Q 28, and M1 particles, respectively. We used NIH Image 1.54 to compare the relative amount of M1 protein detected in each sample. (B) Relative amounts of M1 and VSV-G proteins detected in culture supernatants of Sf9 cells infected with VSV-G/Q or VSV-G/HA/Q. Equivalent numbers of Sf9 cells (5×10^6) were infected with either VSV-G/Q or VSV-G/HA/Q quadruple recombinants (MOI, 5). Culture supernatants were concentrated, and the relative amounts of M1 and VSV-G or VSV-G/HA chimera were measured as described above. Lanes 1 and 2 show the M1 and VSV-G or VSV-G/HA chimeric proteins released from Sf9 cells infected with VSV-G/Q or VSV-G/HA/Q quadruple recombinants, respectively.

We further investigated the interactions among the influenza virus structural components by using single-gene baculovirus recombinants. Infection of Sf9 cells with a monovalent M1 recombinant revealed that matrix protein is able to interact with the plasma membrane and cause budding of vesicular particles from the cell surface. Similar membrane structures were released from insect cells when infected with a baculovirus recombinant that expressed the matrix protein of VSV (11, 16), suggesting that these proteins with analogous functions and derived from closely related viral families also share similar functional properties when expressed in Sf9 cells.

In influenza virus-infected cells, the M1 protein associates with the RNPs in the process of transport and viral assembly. In this case, these other influenza virus structures are not present, yet budding occurs. We conclude that the membrane-associated M1 matrix protein is somehow able to direct the budding and release of vesicular particles from the cell surface.

It is reasonable to question whether matrix protein would be able to drive vesicle formation in mammalian cells or whether this property is only seen in insect cells because of their cell membrane composition. Recently published work has given a clear answer to this question, showing that the expression in COS-1 cells of the M1 protein in the absence of other influenza virus proteins suffices for the formation and release of spikeless vesicles. Similarly, studies with the M protein of VSV demonstrated that this ability was also present in both cell types. In addition, expression of matrix M and NP proteins of parainfluenza virus in mammalian cells led to the formation and release of VLPs (2). Therefore, it is not unexpected that the influenza virus M1 protein demonstrates similar properties in mammalian cells (8).

However, expression of influenza virus M1 and NP proteins in mammalian cells directed by a Semliki Forest virus recombinant did not demonstrate the formation and release of VLPs or association of these two proteins (29). These contrasting results with M1 and NP of influenza and parainfluenza viruses may reflect intrinsic structural and functional differences between these two proteins or that the different expression system may interfere with their interactions. Our results, as well as the results of Gómez-Puertas et al. (8), clearly showed that M1 protein is able to induce particle formation; we do not know, however, why M1 protein expressed by Semliki Forest virus did not lead to vesicle formation.

In view of the fact that four influenza virus proteins were sufficient for the formation and release of VLPs, we asked whether surface glycoproteins of other enveloped viruses could be assembled and released as chimeric influenza VLPs. In pursuit of this goal, we generated two new quadruple baculovirus recombinants that carried, in addition to the influenza virus genes NA, M1, and M2, the VSV surface glycoprotein G. In one construct, we completely replaced the HA gene with a full-length VSV-G, and in the other we made an HA/G chimera which contained the transmembrane region and cytoplasmic tail of HA and the ectodomain of VSV-G. Both of these recombinant viruses were able to drive the expression of the four proteins (M1, M2, NA, and VSV-G), which were also secreted into the medium and sedimented with similar patterns to that of the wild-type VLPs when analyzed by gradient centrifugation.

The exchange of the cytoplasmic tail and transmembrane domain of VSV-G for those of the HA protein enhanced the incorporation of the chimeric glycoprotein into the VLP. This result suggests that interaction of either the cytoplasmic tail or the transmembrane domain or both with the matrix protein are the basis for higher recruitment of glycoprotein into the VLPs. The fact that deletion of the cytoplasmic tail of the HA (10) does not preclude its incorporation into the influenza virion particles suggests that the transmembrane domain also may establish contact with the matrix protein, contributing in the

sorting and assembly process. Alternatively, it could be that the HA and M1 interaction may not be absolutely required for budding from the cell surface.

Incorporation of the wild-type VSV-G protein into particles raises the question of how and where this surface glycoprotein associates with the VLPs. Perhaps matrix protein M1 associates with the plasma membrane and facilitates particle formation from areas with specific biochemical composition (lipid rafts) which favor accumulation of VSV-G. Therefore, colocalization of the influenza virus structural components and G protein may account for its passive incorporation into the VLPs. This simple reasoning may also explain the well-documented passive incorporation of unrelated glycoproteins on the surface of enveloped viruses (pseudotyping).

Our data strongly support the notion that the matrix protein plays a central role in virus assembly and release. The amount of M1 protein associated with virion particles, therefore, may be assumed to reflect the relative efficiency with which these particles are formed and released. Our experiments measuring the relative amount of M1 protein released with influenza virus, HA/Q28 VLPs, and M1 particles indicated that M1 associated with both of the particles to a greater extent than that found in influenza virions. Considering the relative amounts of M1 protein immunoprecipitated from influenza virions and VLPs, we assume that VLP formation and release is a frequent event. However, we cannot determine whether the distribution of M1 protein among the VLPs is similar to that in influenza virus. Although this approach does not permit us to quantify VLP yield, it does help to assess the efficiency with which M1 protein that is associated with particles exits the cells. Incorporation of the VSV-G/HA chimeric glycoprotein onto the surface of the VLPs appears to be greater than wild-type VSV-G when equivalent numbers of cells are infected with either of the quadruple baculovirus recombinants (VSV-G/HA/Q or VSV-G/Q). Similarly, the relative amount of M1 protein associated with particles is higher with the chimeric construct than the wild-type VSV-G construct. This result suggests that the HA cytoplasmic tail and transmembrane domains not only enhance recruitment of the chimeric glycoprotein onto the surface of the particle but also increase the relative amount of M1 protein released. It seems reasonable to conclude that optimal interaction between the surface glycoproteins and underlying matrix protein may enhance the efficiency of particle assembly and exit.

This novel approach for the assembly of influenza virus particles has great potential for the design of vaccines against new influenza virus variants. Even extremely dangerous subtype antigen combinations, such as H1N1 (from the 1918 Spanish flu) or an HA-NA combination with pandemic potential, could be incorporated into VLPs without concern about the implications of releasing genes that have not circulated in humans for several decades. Furthermore, the feasibility of incorporating heterologous glycoproteins onto the surface of the VLPs makes this approach attractive not only as a vaccine delivery system but also as one that will allow for the targeting of specific cell types based upon the proteins incorporated into the surface of the VLPs.

In summary, we have shown that wild-type and chimeric influenza VLPs can be assembled and released from the surface of Sf9 cells following expression of only four viral proteins. We also demonstrated that matrix protein M1 by itself is able to drive the release of vesicular particles. This approach will allow us to further study viral morphogenesis, and it holds promise for creating novel vaccines for influenza virus and possibly for other pathogens.

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