

## Basis for Selective Incorporation of Glycoproteins into the Influenza Virus Envelope

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**The ability of mutant or chimeric A/Japan hemagglutinins (HAs) to compete for space in the envelope of A/WSN influenza viruses was investigated with monkey kidney fibroblasts that were infected with recombinant simian virus 40 vectors expressing the Japan proteins and superinfected with A/WSN influenza virus. Wild-type Japan HA assembled into virions as well as WSN HA did. Japan HA lacking its cytoplasmic sequences, HA<sup>tail-</sup>, was incorporated into influenza virions at half the efficiency of wild-type Japan HA. Chimeric HAs containing the 11 cytoplasmic amino acids of the herpes simplex virus type 1 gC glycoprotein or the 29 cytoplasmic amino acids of the vesicular stomatitis virus G protein were incorporated into virions at less than 1% the efficiency of HA<sup>tail-</sup>. Thus, the cytoplasmic domain of HA was not required for the selection process; however, foreign cytoplasmic sequences, even short ones, were excluded. A chimeric HA having the gC transmembrane domain and the HA cytoplasmic domain (HgCH) was incorporated at 4% the efficiency of HA<sup>tail-</sup>. When expressed from simian virus 40 recombinants in this system, vesicular stomatitis virus G protein with or without (G<sup>tail-</sup>) its cytoplasmic domain was essentially excluded from influenza virions. Taken together, these data indicate that the HA transmembrane domain is required for incorporation of HA into influenza virions. The slightly more efficient incorporation of HgCH than G or G<sup>tail-</sup> could indicate that the region important for assembling HA into virions extends into part of the cytoplasmic domain.**

The formation of a membrane domain in which certain proteins are concentrated and others are excluded is a process central to cellular organization. The formation of secretory or endocytic vesicles, the development of cell surface polarity, and the separation of internal membranes into separate organelles all involve this process. Just as viruses have been remarkably useful systems for studies of DNA replication, transcription, and RNA processing and for the assembly of macromolecular protein complexes, enveloped viruses are excellent models for the formation of membrane domains that have a simple, defined composition. Like many enveloped viruses, type A influenza viruses assemble at the plasma membrane. During the maturation process, the influenza virus hemagglutinin (HA), neuraminidase (NA), and M2 glycoproteins are concentrated in a patch of plasma membrane that effectively excludes cellular proteins (reviewed in reference 4). In polarized epithelial cells, influenza virus buds selectively from the apical domain of the plasma membrane (29), which is the site of insertion of the virus glycoproteins (17, 28, 30). This observation is consistent with the hypothesis that enveloped viruses bud at the site at which their virus glycoproteins are most concentrated (32), an idea that implies that a recognition event between one or more internal proteins and the virus glycoproteins is required to nucleate virion assembly. Indeed, mutations in either the influenza virus HA or M1 proteins can completely block virus budding (5).

Viruses appear to differ in their requirements for incorporating envelope glycoproteins. The envelopes of retroviruses are relatively permissive for foreign proteins (8, 23, 35, 37-39), and retroviruses in some cases can bud from the plasma membrane in the absence of the envelope glycopro-

tein gene (6). In contrast, alphaviruses, in which the surface glycoproteins adopt a defined symmetry in relation to an interior protein capsid (11), do not incorporate foreign proteins, apparently as the result of an early association between nucleocapsids and the glycoproteins (19, 41). Influenza viruses are intermediate in this respect, failing to incorporate vesicular stomatitis virus (VSV) G protein during mixed infections (40) but apparently able to form phenotypically mixed viruses with Newcastle disease virus (14). The basis for this selective incorporation of glycoproteins into the influenza virus envelope has not been previously established.

The cytoplasmic sequences of HAs of the 14 subtypes of influenza A viruses show remarkable conservation (9, 26, 33). Because the HA cytoplasmic sequences are not necessary for HA biosynthesis or for its functions of binding sialic acid and inducing membrane fusion, it was suggested that the conserved residues form the binding site for an internal influenza virus protein, probably M1 (9). Recently Simpson and Lamb (33) have shown that HA lacking its cytoplasmic sequences can be incorporated into noninfectious virions. Therefore, the conserved cytoplasmic sequences of HA are not required for incorporation of HA into the virus but are necessary for infectivity. A possible role of the HA transmembrane sequences in the incorporation of HA into influenza virus had not been previously investigated.

Not only are the interactions among influenza virus proteins that result in virion assembly unknown, but the mechanism by which host proteins are excluded from the viral envelope is equally obscure. To investigate the basis for exclusion of foreign proteins from influenza virions, we have compared the abilities of wild-type, mutant, and chimeric A/Japan HAs containing foreign cytoplasmic and/or transmembrane domains to be incorporated into A/WSN influenza virus. Under our conditions, the mutant Japan HAs are expressed from cDNAs and, like cellular glycoproteins, are

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present at the cell surface when influenza virus proteins begin to assemble into virions. Like cellular proteins, they are initially present at a high concentration relative to the influenza virus HA but at later times of infection must compete with the wild-type WSN glycoprotein for inclusion in virions. This experimental system allows us to investigate the possibility of both a requirement for a positive signal for incorporation into the influenza virus envelope and a mechanism for excluding proteins containing incompatible transmembrane or cytoplasmic sequences.

## MATERIALS AND METHODS

**Virus stocks.** The recombinant proteins used in these studies have been characterized previously (21, 34). Unless designated otherwise, enzymes for recombinant DNA work and reagents for cell culture were from GIBCO BRL (Gaithersburg, Md.) and other reagents were from Sigma Chemical Co. (St. Louis, Mo.). CV-1 cells (American Type Culture Collection, Rockville, Md.) between passages 21 and 24 were used for all experiments. Recombinant simian virus 40 (SV40) stocks were prepared as described previously (28), with a few modifications. At 36 h after transfecting CV-1 cells with SV40 vector and helper virus DNA, an equal number of untransfected cells was added to each dish of transfected cells to supply healthy cells for subsequent rounds of virus growth. After 3 to 5 days, the cells were frozen and thawed three times and the medium and broken cells were collected and vortexed. This was frozen as seed virus stock. To expand the titer of the seed virus to the levels required for experimental work, each stock was used undiluted to infect fresh cells as follows. Subconfluent CV-1 cells were removed from culture plates with trypsin and were centrifuged for 3 min at  $700 \times g$  through 10 ml of Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, N.Y.) containing 10% fetal calf serum. Cells were resuspended at a concentration of  $3 \times 10^6$  CV-1 cells in 200  $\mu$ l of seed virus stock and were incubated on ice or at room temperature for 30 to 45 min. DMEM containing 10% Serum Plus (Hazleton, Lenexa, Kans.) was added to the infected cell pellet, and the cells were seeded into culture plates at 50% of maximum cell density. Viruses were harvested as described above when cytopathic effect had destroyed half of the cell monolayer, typically on the third day after infection. Recombinant SV40 stocks prepared by this method were uniformly of high titer as determined by immunofluorescence assays for recombinant proteins produced in CV-1 cells infected with serial dilutions of the virus stocks.

**Infections for experiments.** For double infections, CV-1 cells were infected in suspension with recombinant SV40s as described above. After 32 h, WSN influenza virus at a multiplicity of infection (MOI) of 10 was allowed to adsorb to the SV40-infected cells in a small volume for 1.5 h at 37°C. Single infections with WSN were performed on CV-1 cells 32 h after a mock infection in which they were manipulated exactly as for infection with recombinant viruses. In some experiments with doubly infected cells, NA from *Vibrio cholerae* (Boehringer Mannheim) was present at 4 mU/ml in the medium. The presence or absence of NA had no effect on the release of viruses from the cells, and so use of NA was discontinued.

**Antisera.** Anti-WSN and anti-VSV antisera were prepared in rabbits injected subcutaneously with either gradient-purified, whole WSN virus or gradient-purified VSV emulsified in Freund's complete adjuvant. Rabbits were injected

again after 3 weeks with the same virus in incomplete adjuvant and were bled 1 week after the second injection. Subsequent injections were performed at monthly intervals, and serum was collected a week after each injection. A similar protocol was used to produce antibodies specific for Japan virus, except that the virus glycoproteins were extracted from the virus (20) for use as antigen. This glycoprotein preparation was contaminated with the Japan NP protein and produced an antiserum that reacted with Japan HA, NA, and NP proteins.

**Quantification of radioactive proteins from cells and from virus.** For labeling with radioactive amino acids, cells were preincubated in DMEM lacking methionine and cysteine for 20 min and were labeled in this medium with 100  $\mu$ Ci of Tran<sup>35</sup>S label (ICN Radiochemicals, Irvine, Calif.) per ml for the periods described below. Recombinant proteins produced by our SV40 late replacement vectors were first detected 24 h after infection. Thus, for most double-infection experiments, radioactive labeling of cells infected with SV40 vectors was begun 24 h postinfection (hpi). At 32 hpi, cells were either superinfected with WSN virus or mock infected, in the case of single infection, with SV40 vectors. During the 1.5-h period for adsorption of WSN (and during the mock infection), cells were cultured in normal medium lacking radioactive amino acids. This nonradioactive inoculum was removed, and the cells were washed three times with warm DMEM and then were incubated for 30 min at 37°C in DMEM lacking methionine or cysteine. The doubly infected cells were then labeled with Tran<sup>35</sup>S label for the next 6 to 8 h. Cells singly infected with SV40 were labeled for the first 6-h period followed by a chase in nonradioactive medium for 2.5 h exactly as the doubly infected cells were. This was done to measure the expression of Japan HAs or G proteins at the cell surface at the time of superinfection. Experiments in which the initial labeling period was for 3 h beginning at 29 hpi gave similar results. Cells infected only with WSN were cultured identically to those infected with SV40s but were labeled only for the 6 h after the 1.5-h period for WSN adsorption. For experiments in which amounts of cell-associated proteins were measured, cells were lysed in 50 mM Tris (pH 8.0) containing 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), and 0.1  $\mu$ U of aprotinin per ml (Boehringer Mannheim) at the end of the labeling period. The lysates were centrifuged for 10 min at  $14,000 \times g$  to remove large material, and the supernatant fraction was immunoprecipitated overnight at 4°C or for 3 h at 37°C with the appropriate antiserum and protein A-Sepharose (Pharmacia, Uppsala, Sweden). Proteins in immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The resulting gels were processed for fluorography and exposed to X-Omat film (Kodak, Rochester, N.Y.) at -80°C. Images on the films were quantified by laser scanning densitometry (Molecular Dynamics, Sunnyvale, Calif.) as described previously (2).

In many experiments, radioactive viruses were collected from culture medium of cells infected and labeled as described above. For this, medium was first clarified by centrifugation at  $10,000 \times g$  for 20 min and then either was layered over a 22% sucrose cushion and centrifuged for 1.5 h at  $210,000 \times g_{av}$  or was layered at the top of a 20 to 60% discontinuous gradient of sucrose (0.5 ml at 20%, 2.0 ml at 30%, 1.5 ml at 40%, and 0.5 ml at 60%) in 1 mM Tris-10 mM EDTA-100 mM NaCl (pH 7.8) and was centrifuged at  $197,000 \times g_{av}$  for 1.5 h at 4°C. Virus pelleted through a sucrose cushion was lysed with cell lysis buffer and then was diluted with NET/Gel (50 mM Tris [pH 8.0], 0.5% Nonidet

TABLE 1. Comparison of surface expression of recombinant and viral glycoproteins

Protein	Carboxy-terminal sequence <sup>a</sup>	Relative surface expression <sup>b</sup>	% in medium <sup>c</sup>
A/WSN HA	<u>ILAIYSTVASSLVLLVSLGAI</u> <u>SFFWCS</u> NGSLQCRICI	5.0	NA <sup>d</sup>
Japan HA	<u>ILAIYATVAGSLSLSIMMAGIS</u> <u>SFFWCS</u> NGSLQCRICI	1.0	<1
HA <sup>tail-</sup>	<u>ILAIYATVAGSLSLSIMMAGIS</u> <u>SFFWCSK</u>	1.1	<1
HHgC	<u>ILAIYATVAGSLSLSIMMAGIS</u> <u>SFFWCS</u> RTSQRQRHRR	0.9	<1
HHG	<u>ILAIYATVAGSLSLSIMMAGIS</u> <u>SFFWCS</u> RVGIHLCLIKLKHETKKRQIYTDIEMNRLGK	0.9	<1
HgCH	<u>WVGIGIGVLAAGVLYVTAIVY</u> <u>VVCS</u> NGSLQCRICI	0.6	<1
VSV G	<u>SSIASFFFTIIGLIIGLFLVLR</u> VGIHLCLIKLKHETKKRQIYTDIEMNRLGK	1.0	8
VSV G <sup>tail-</sup>	<u>SSIASFFFTIIGLIIGLFLVLR</u>	1.0	5

<sup>a</sup> Transmembrane (underlined) and cytoplasmic sequences are shown for each protein.

<sup>b</sup> Expression is normalized to that of the Japan HA.

<sup>c</sup> The fraction of the protein recovered from immunoprecipitation of cell culture medium is given as the percentage of labeled protein immunoprecipitated from both culture medium and cells.

<sup>d</sup> NA, Not applicable.

P-40, 0.1% SDS, 10 mM EDTA, 0.1  $\mu$ U of aprotinin per ml, 0.25% gelatin) for immunoprecipitation with the appropriate antiserum. In the case of gradients, fractions of 0.45 ml were collected from the top with a gradient collector. Each fraction was mixed for 10 min with 0.45 ml of lysis buffer containing 2% Nonidet P-40, and then an equal volume of NET/Gel was added. In each case, for double infections samples were immunoprecipitated sequentially with antiserum specific for the protein expressed by the SV40 vector and then with anti-WSN serum. Immunoprecipitates of virus proteins from virions were analyzed as described above for cell lysates.

**Analysis by fluorescence-activated flow cytometry.** CV-1 cells that were infected with vectors expressing G proteins and uninfected controls were labeled in suspension with a 1:200 dilution of anti-G rabbit serum followed by 1:200 dilution of goat anti-rabbit fluorescein isothiocyanate second antibody (Fisher). Cells were analyzed with a FACStar plus (Becton Dickinson), with forward light scatter and fluorescence intensity as sorting parameters to ensure that only single cells were included in the set of data.

**Assays for phenotypic mixing by neutralization of infectivity.** All virus titers were determined by plaque assay on MDCK cells. Confluent MDCK cells were seeded into 12-well dishes at approximately  $6 \times 10^5$  cells per well. For antibody neutralization, virus was incubated at 4°C for 45 min on a platform rocker with anti-Japan, anti-VSV, anti-WSN, or a mixture of anti-WSN and anti-Japan polyclonal antibodies diluted 1:200 in DMEM. Virus was diluted in 10-fold steps in ice-cold DMEM containing the same dilution of antibody. Cells were washed three times with serum-free medium, and then virus was adsorbed to the cells at 37°C for 1.5 h. The inoculum was aspirated, the cells were washed once, and then warm medium was added for 1.5 h. At the end of this incubation, an overlay (0.7% Noble agar in DMEM containing 1  $\mu$ g of trypsin per ml) was applied and plates were incubated in a 37°C incubator with 5% CO<sub>2</sub>. Two days later, plaques were visible and titers were determined.

## RESULTS

Chimeric HA glycoproteins in which the transmembrane and/or cytoplasmic domains of HA were replaced by the analogous regions of either the VSV G protein or herpes simplex virus type 1 gC protein have been characterized previously (21). In addition, forms of the Japan HA and the VSV G protein in which the cytoplasmic sequences were

replaced with a single arginine or lysine were constructed by site-directed mutagenesis (34). The transmembrane and cytoplasmic sequences of these proteins and their names are presented in Table 1. In our nomenclature, chimeric proteins are designated with a character for each of the three topological domains of the protein. Thus, a protein with the HA external domain, the herpes simplex virus type 1 gC transmembrane domain, and the HA cytoplasmic domain is called HgCH.

The cDNAs for the proteins in Table 1 were subcloned into an SV40 expression vector under control of the late promoter, and virus stocks were prepared (9, 12). Because mutations in the cytoplasmic or transmembrane domains of HA may interfere with protein folding and oligomerization (9, 12), the proteins used in this study were tested for their ability to fold and to be transported to the cell surface. Our results (not shown) were in agreement with previous studies

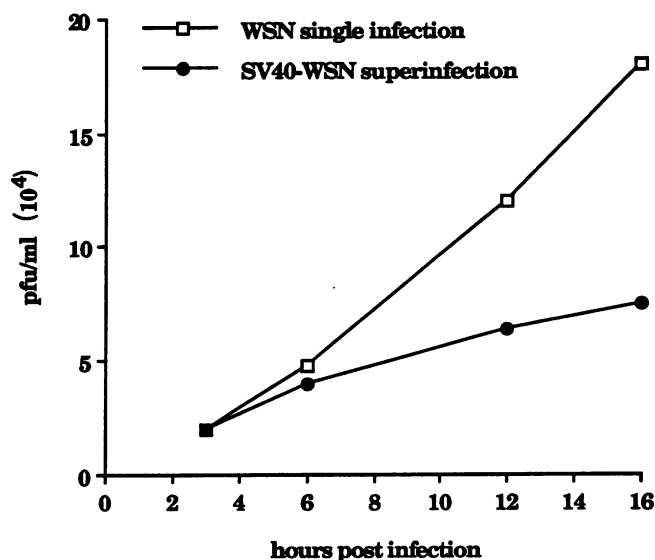


FIG. 1. Growth of influenza A/WSN/33 virus in CV-1 cells. One set of subconfluent CV-1 monolayers was infected with the SV40 vector expressing Japan HA; the other was mock infected. At 32 hpi, both sets of cultures were infected with WSN virus at an MOI of 10. Medium was then collected at 0, 3, 6, 12, and 16 h after the second infection. Virus titers were measured by plaque assay on MDCK cells.

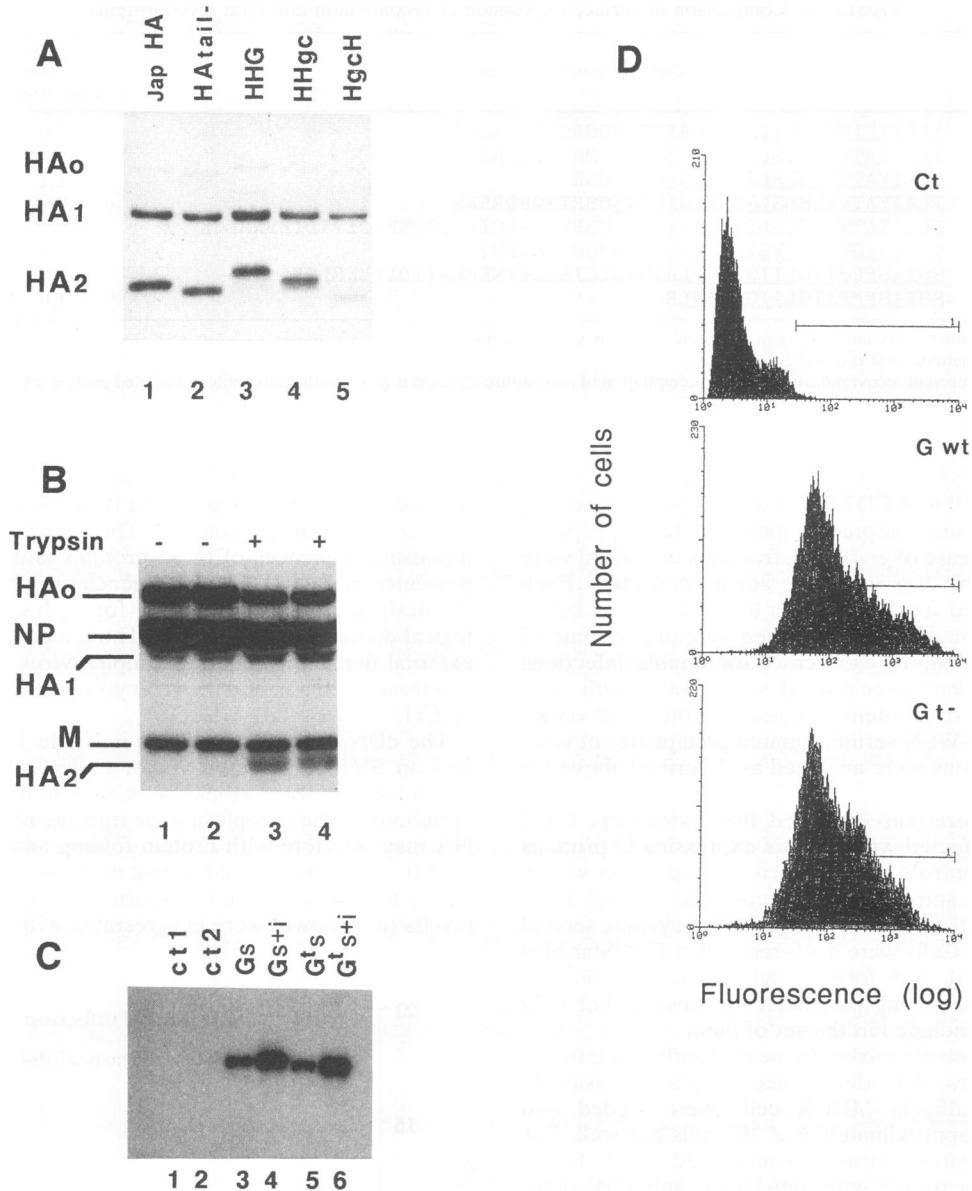


FIG. 2. Comparison of the amounts of proteins expressed by SV40 vectors and by WSN at the surface of CV-1 cells. (A) CV-1 cells were singly infected with recombinant SV40 vectors and were labeled with  $^{35}\text{S}$ -labeled amino acids between 24 and 32 hpi and were chased for 2.5 h in DMEM. Cells were incubated with trypsin on ice for 45 min to cleave HA into its HA1 and HA2 subunits, and the proteins were recovered by immunoprecipitation and analyzed by fluorography. (B) Parallel CV-1 cell cultures were infected with the SV40-Japan HA vector or were mock infected. After 32 h, each was infected with WSN virus, and after 2.5 h each was labeled with  $^{35}\text{S}$ -labeled amino acids for 6 h. One singly infected culture and one doubly infected culture were treated with trypsin at  $4^\circ\text{C}$ . HAs were then immunoprecipitated with anti-WSN serum. Lanes 1 and 3, WSN-infected cells; lanes 2 and 4, SV40- and WSN-superinfected cells. HAo is the uncleaved HA. (C) Cells expressing G or  $G^{\text{tail-}}$  ( $G^t$ ) were labeled beginning at 27 hpi (G) or 30 hpi ( $G^t$ ) for 3 h and were chased in DMEM for 3 or 5 h, respectively. G proteins were recovered by surface immunoprecipitation and were analyzed by PAGE. s indicates samples bound by antibodies at the cell surface. s+i indicates standard immunoprecipitation of lysed samples to recover total G proteins. As controls, WSN-infected cells (Ct1) or SV40-G-infected cells (Ct2) were incubated with anti-G serum for surface immunoprecipitation. Lysates of these cells were mixed with lysates containing radioactive G protein to determine whether antibodies adsorbed nonspecifically to cells could influence the assay by binding labeled G proteins after cell lysis. (D) At 27 hpi (G) or 30 hpi ( $G^{\text{tail-}}$ ), cells expressing recombinant G proteins were analyzed by fluorescence-activated flow cytometry. Profiles showing cell number as a function of the log of fluorescence intensity are shown for uninfected cells (Ct) and cells expressing wild-type G (G wt), and  $G^{\text{tail-}}$  ( $G^t$ ). The horizontal lines designate the region containing 99% of the cells expressing either G protein.

demonstrating that removing the cytoplasmic domain or replacing the transmembrane domain of HA or truncating the cytoplasmic domain of G does not block transport of the mutant proteins to the cell surface, although in some cases

the rate of transport is reduced (7, 33, 36), and HAs with foreign transmembrane domains are less able to fold normally (21, 31).

Conditions were established so that essentially all cells in

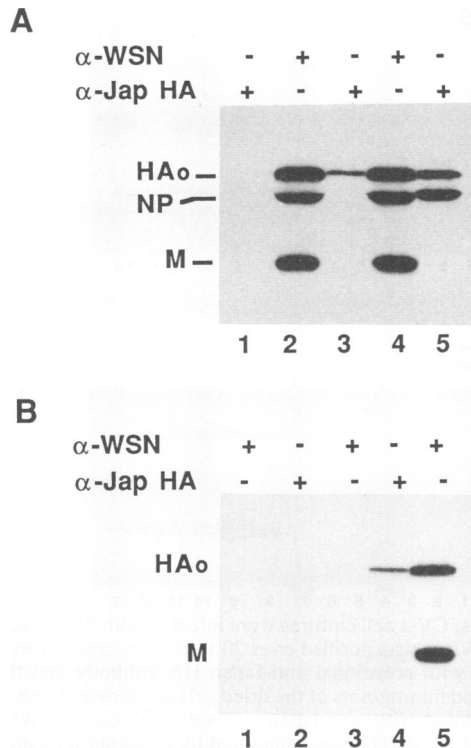


FIG. 3. Incorporation of Japan HA into WSN virus. CV-1 cells were infected with SV40-Japan virus vector (controls were mock infected). (A) After 24 h, samples were labeled with  $^{35}\text{S}$ -labeled amino acids as described below. Infected and mock-infected monolayers were then superinfected with WSN virus at an MOI of 10. After an initial infection of 1.5 h, samples were labeled again for 8 h. Viruses were collected from culture supernatants by centrifugation through a sucrose cushion, were lysed with detergent, and were immunoprecipitated with either anti-Japan or anti-WSN polyclonal antibodies. (B) The supernatant of each immunoprecipitation was precipitated again with the opposite antibody. Lanes: 1, SV40 Japan HA only, labeled for 8 h beginning at 24 hpi; 2, A/WSN virus only, labeled for 8 h beginning at 1.5 hpi; 3, SV40 Japan HA labeled and then superinfected with WSN 32 h after the SV40 vector infection and not labeled further; 4, SV40 Japan HA, superinfected with WSN 32 hpi and then labeled; 5, SV40 Japan HA, labeled, superinfected with WSN 32 hpi, and then labeled again. HAo, uncleaved HA; NP, nucleoprotein; M, matrix protein.

a culture were doubly infected with recombinant SV40s and with WSN influenza virus. SV40 has a host range limited to a few simian species, and the best host cell line available is the CV-1 line of African green monkey fibroblasts. A time course of the production of influenza viruses by CV-1 cells singly infected with WSN was compared with that produced when CV-1 cells were first infected with recombinant SV40 viruses and 32 to 35 h later were superinfected with WSN virus (Fig. 1). Conditions optimum for SV40 infection require that CV-1 cells be subconfluent and actively growing, whereas in standard influenza virus infections cells are usually at maximum density. Correcting for the much lower cell density, singly infected subconfluent CV-1 cells produced as much influenza virus during the first 6 h of infection as a good virus host such as MDCK cells (data not shown). Little difference was observed in influenza virus production in singly or doubly infected cells during this 6-h interval, indicating that neither the SV40 infection nor the presence of

recombinant proteins interfered with production of infectious influenza viruses. After this period, the rate of production of influenza virus in SV40-infected cells slowed dramatically; even singly infected CV-1 cells produced titers of virus, on a per cell basis, of less than 10% of those observed in MDCK cells. Influenza virus production was also impaired when SV40-infected cells were superinfected with WSN virus at periods much later than 35 h after SV40 infection (not shown). Since there is an initial eclipse phase of several hours during which no influenza virus is produced, our experiments measured only the first hours of virus production. This period was, however, sufficient to give titers of virus greater than  $10^4$  PFU/ml, which allowed us to confidently detect pseudotype viruses that might be only 1% of the virus harvested.

To be able to compare the relative abilities of Japan HA and VSV G protein to be incorporated into WSN virions, it was important that our recombinant virus stocks produced similar amounts of protein at the cell surface under the conditions of our experiments. Because the different HA mutants to be studied had different rates of transport to the cell surface and, in the case of HgCH, a faster rate of degradation, the period after SV40 infection when cells were superinfected with WSN was adjusted so that the level of each protein at the cell surface was similar at the period when influenza virions began to form (Table 1). The largest difference in rate of transport was seen between the G and  $G^{\text{tail-}}$  proteins. To allow for this difference, in parallel experiments cells were infected with the  $G^{\text{tail-}}$  SV40 vector 3 h earlier than with the vector expressing G. Comparison of the amounts of each type of protein at the cell surface by flow cytometry indicated that, under these conditions, the amount of protein at the cell surface at 27 (G) or 30 ( $G^{\text{tail-}}$ ) hpi differed by less than 5% (Fig. 2D). In experiments employing radioactive labeling of doubly infected cells, the period of single infection with the vector expressing  $G^{\text{tail-}}$  was extended by 3 h, the labeling time was increased by 1 h, and the chase was increased by 2 h to allow for the slower transport of labeled  $G^{\text{tail-}}$  protein to the cell surface.

To compare the strengths of the recombinant virus stocks expressing forms of Japan HAs, cells singly infected with viruses producing each of the proteins to be tested were labeled with  $^{35}\text{S}$ -labeled amino acids under the same conditions as would be used for double infections (Fig. 2A). HA proteins were then chased in the absence of radioactive amino acids for 2.5 h, and the proportion of proteins reaching the cell surface was determined by cleavage into HA1 and HA2 with trypsin added to the medium on ice. The amount of the various Japan HAs produced by recombinant viruses differed by less than twofold. After a 2.5-h chase, essentially all of the wild-type or mutant Japan HAs had been transported to the cell surface. In a double infection, WSN virus inhibited protein expression by CV-1 cells and recombinant vectors beginning approximately 4 h after superinfection (not shown). Thus, 6 to 8 h was chosen as the interval for WSN growth in doubly infected cells, a period longer than required for all labeled Japan proteins to reach the cell surface after their synthesis had been inhibited.

To determine the relative amounts of HAs produced in cells infected with WSN under the conditions used for double infections, CV-1 cells were infected with WSN virus alone or at 32 h after infection with the vector producing Japan HA. Both sets of cells were labeled for 6 h beginning after adsorption of WSN, and then WSN proteins were recovered by immunoprecipitation. WSN virus produced fivefold more HA at the cell surface than did the SV40

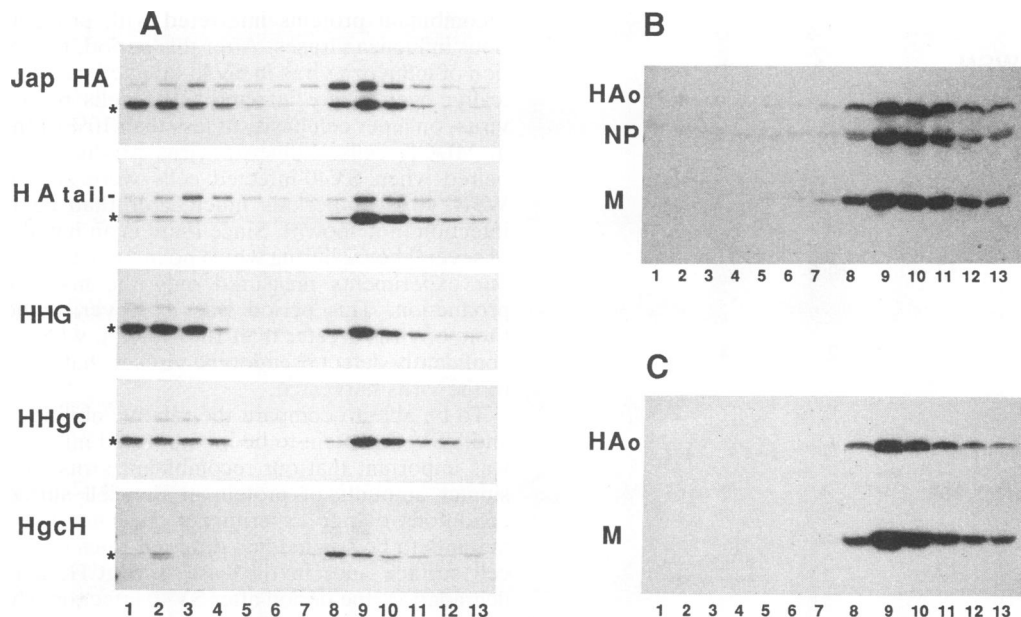


FIG. 4. Incorporation of Japan HA mutants into phenotypically mixed WSN virus. CV-1 cell cultures were infected with SV40 vectors and superinfected with WSN virus. HA proteins were labeled as described for Fig. 3. Virus was purified over 20 to 60% discontinuous sucrose gradients, and fractions were collected and sequentially immunoprecipitated first with polyclonal anti-Japan HA antibody and then with polyclonal anti-WSN antibody. The immunoprecipitates were analyzed by PAGE, and fluorograms of the dried gels are shown. Lane 1 is from the top of the gradient. (A) Phenotypically mixed influenza virus immunoprecipitated with anti-Japan antibody. (B) Control WSN virus immunoprecipitated with anti-WSN virus antibody. (C) For each Japan HA mutant, WSN HA was quantified by a second immunoprecipitation with polyclonal anti-WSN antibody. Only the second immunoprecipitation of the Japan HA (top, A) is shown. Asterisks indicate the NP protein that cross-reacted with the anti-Japan virus-glycoprotein polyclonal antibody. HAo, uncleaved HA; M, matrix protein.

vectors under the conditions employed (compare Fig. 2B, lane 3, with 2A). No reduction in WSN protein synthesis was observed in doubly infected cells (Fig. 2B). Thus, the SV40 infection had no effect on the production of WSN proteins over this period.

To compare the amounts of radioactive G proteins at the cell surface, CV-1 cells expressing G were labeled for 3 h and were chased for 3 h. Cells expressing G<sup>tail-</sup> were labeled for 4 h and were chased for 5 h. Anti-G serum was added to the cells at 4°C for 45 min, and the cells were washed three times. The G proteins were then recovered by immunoprecipitation with protein A-Sepharose (Fig. 2C). As controls for the possibility that antibodies to G might stick to the cell surface and bind G after cell lysis, unlabeled cells either infected with WSN viruses (Ct1) or infected with SV40-G (Ct2) were incubated with anti-G antibodies and, after washing, were lysed and mixed with lysates of cells containing radioactive G proteins. These mixed samples were immunoprecipitated with protein A-Sepharose and analyzed. As shown in Fig. 2C, there was no subsequent redistribution of anti-G antibodies in either control (lanes 1 and 2). Similar amounts of G or G<sup>tail-</sup> were detected at the cell surface in this assay (lanes 3 and 5), and total amounts of each protein produced were similar (lanes 4 and 6). In addition to measuring the protein present at the cell surface, the culture medium from each of the experiments shown in Fig. 2 was also immunoprecipitated to determine the amount of protein shed from the cell surface. This was done to control for the possibility that a shed protein might adsorb to the outside of influenza virions and give a false appearance of incorporation into the virus envelope. Of the proteins employed, only G and G<sup>tail-</sup> were shed into the medium to any extent (Table

1). The shedding of G proteins from the cell surface has been well documented (3, 15, 18, 22).

Mixed infection of cells with type A influenza viruses having HAs of different subtypes does not produce mixed trimeric HAs, probably because of the large number of sequence differences in their external domains (1). However, phenotypically mixed virus is produced by cells coinfecting with influenza viruses with HAs as divergent as types A and B (13, 16). Thus, we expected Japan HA trimers to be efficiently incorporated into WSN virions in doubly infected cells. To detect this biochemically, we compared the amounts of radioactive Japan HA in CV-1 cell culture supernatants when cells were singly infected with the SV40-Japan HA vector, singly infected with WSN virus, or doubly infected. Cells were labeled with <sup>35</sup>S-labeled amino acids in three different ways to determine how much of the protein produced by SV40 vectors was made before or after the superinfection with WSN. This was done either by labeling continuously from 24 h after infection with SV40 vectors, by labeling only from 24 h to 32 h when the WSN infection was begun, or by labeling only during the period after the WSN infection. Four cultures of CV-1 cells in suspension were infected with SV40-Japan HA vector, and one was mock infected. The cells were seeded into dishes, and at 24 hpi, three of the four infected cultures were labeled with 100 μCi of Tran<sup>35</sup>S label per ml for 8 h (Fig. 3, lanes 1 and 5). The fourth infected culture would be labeled later, only after WSN infection. At 32 hpi, the mock-infected monolayer, the unlabeled SV40 vector-infected monolayer, and two of the three labeled SV40-infected cultures were superinfected with WSN virus at an MOI of 10 PFU per cell. The remaining labeled SV40-infected culture was left without



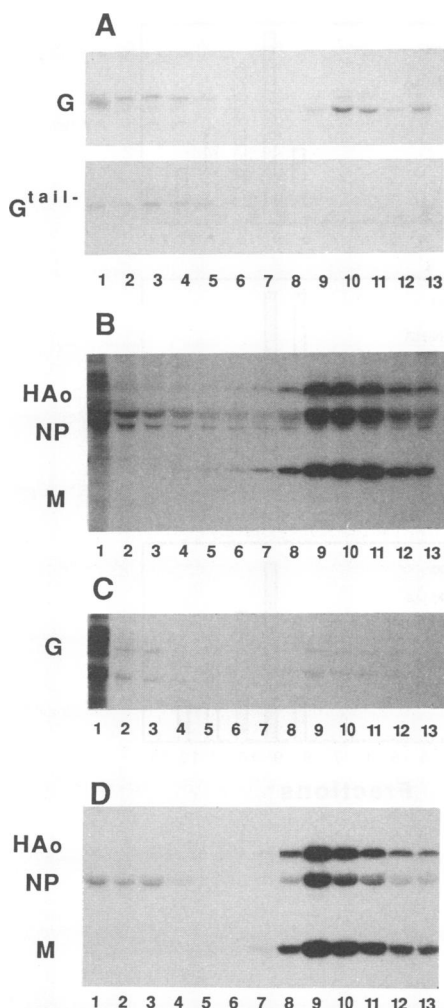


FIG. 5. Incorporation of G or  $G^{\text{tail-}}$  into phenotypically mixed WSN virus. The amount of G or  $G^{\text{tail-}}$  incorporated into WSN virus was measured by the protocols described in the legends to Fig. 3 and 4, except that  $G^{\text{tail-}}$  infection and labeling times were corrected as described in Materials and Methods. Gradient fractions were precipitated sequentially with anti-VSV virus polyclonal antibody (A) and anti-WSN virus antibody (B). As a control, two CV-1 cell cultures were infected separately, one with SV40-G, the other with WSN, and the proteins were labeled according to the protocols used for superinfections. The medium of the culture infected with SV40-G was harvested at 38 hpi, and the medium of the culture infected with WSN was harvested at 6 hpi. Half of each culture supernatant was mixed together and incubated at 37°C for 2 h. Virus from mixed and unmixed culture supernatants was purified on sucrose gradients and sequentially immunoprecipitated with anti-VSV (C) and anti-WSN (D) antibodies. HA<sub>o</sub>, uncleaved HA; NP, nucleoprotein; M, matrix protein.

WSN infection as a control for any release of Japan HA into the culture medium that was unrelated to the WSN infection (lane 1). This singly infected culture and one of the two labeled doubly infected cultures were labeled continuously throughout the experiment (lanes 1 and 5). The other cultures were not labeled during the 1.5-h absorption of WSN or during an additional 1 h in DMEM lacking methionine and cysteine. One of these doubly infected cultures was cultured for the rest of the experiment without additional labeling (lane 3) so that only the incorporation into virus of Japan

HAs produced before the WSN infection was measured. The culture singly infected with WSN (lane 2) and the doubly infected culture that had not been labeled initially (lane 4) were labeled for 6 h. Culture supernatants were then collected, and viruses were isolated from them by centrifugation through a sucrose cushion (see Materials and Methods). Virus pellets were lysed and immunoprecipitated sequentially, first with either anti-Japan HA or anti-WSN polyclonal antibodies (Fig. 3A), and then the supernatant of each precipitation was immunoprecipitated again with the other antibody (Fig. 3B). Japan HA was detected in the medium of cells only if they had been superinfected with WSN virus (compare lanes 1 to 3 and lane 5). When the labeling period was continuous, Japan HA was approximately 40% of the total HA recovered from virus pellets. When doubly infected cells were labeled only before superinfection (lane 3) or only for the last 6 h of infection (lane 4), relatively little labeled Japan HA was recovered. Thus, most of the Japan protein appearing in virions was synthesized during the period of superinfection and prior to the inhibition of host cell synthesis by WSN. Because the ratio of Japan HA to WSN HA at the cell surface by the end of the labeling period was 1 to 5, the incorporation of Japan HA was quite efficient, and it is possible that the very first influenza viruses produced contained predominantly Japan HA.

**Incorporation of Japan HA and mutants, VSV-G, and VSV- $G^{\text{tail-}}$  into WSN virus.** Double infections were next performed with WSN and SV40 vectors expressing each of the proteins shown in Table 1. Influenza viruses resulting from each infection were centrifuged through 20 to 60% discontinuous sucrose gradients. Gradient fractions were collected, mixed with detergent to dissociate the viral proteins, and immunoprecipitated sequentially, first with anti-Japan serum followed by a second precipitation with anti-WSN serum. The results are shown in Fig. 4. Panel A presents autoradiograms of the first immunoprecipitations. HA proteins were present in two regions of the gradient. Fractions 8 to 11 contained the bulk of the influenza virus peak, containing HA and NP proteins. The cross-reactivity of the anti-Japan serum with the WSN NP protein was expected, because type A influenza viruses are grouped according to the cross-reactivity of their NP proteins. That influenza virus was indeed present in fractions 8 to 11 was shown by a parallel control experiment (panel B) in which the culture supernatant from cells infected only with WSN virus was centrifuged over a similar gradient, collected in fractions, and immunoprecipitated with anti-WSN antibody. WSN HA, NP, and M proteins were each present in fractions 8 to 13 with the main peak in fractions 8 to 11. The same control was performed for each of the gradients containing samples from doubly infected cells. Fractions of each of the five gradients shown in panel A were immunoprecipitated a second time with anti-WSN serum with similar results, one of which is shown in panel C. The WSN NP protein was often absent from these fractions, having been removed by the anti-Japan serum in the first immunoprecipitation. Comparing fractions 8 to 13 of each of the gradients in panel A, it is obvious that the wild-type Japan HA incorporated fairly well into virus but mutant HAs containing foreign cytoplasmic or transmembrane domains (HHG, HHgC, and HgCH) incorporated poorly. HA<sup>tail-</sup> lacking the cytoplasmic domain was detected in reduced amounts compared with wild-type Japan HA but was much more abundant than were HAs of the other mutants. In each of the doubly infected samples, WSN NP and Japan HAs were also detected in fractions of lower density at the top of the

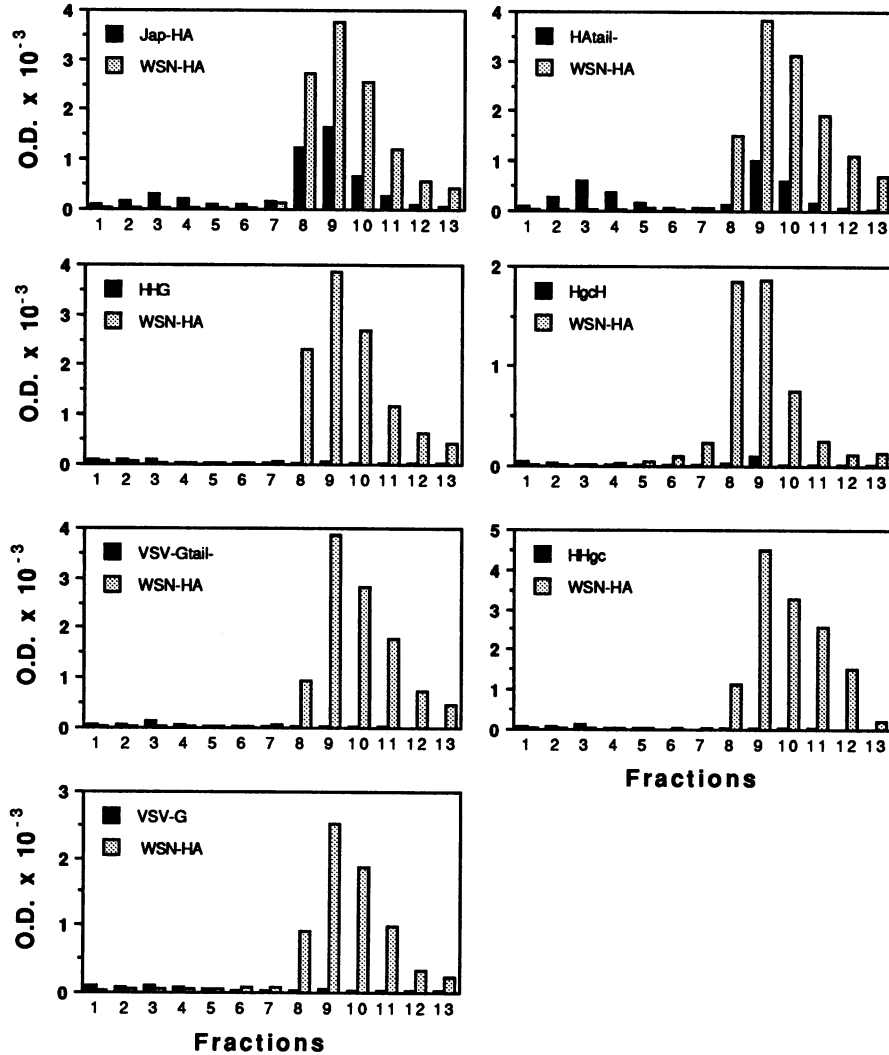


FIG. 6. Analysis of the rate of incorporation of various mutants into WSN virus. Fluorograms from Fig. 4 and 5 were quantified by laser scanning densitometry, and the amount of a mutant HA or G protein was compared with that of WSN HA recovered from the same fraction. O.D., optical density.

gradient (fractions 1 to 4). WSN M protein was never detected in these fractions, nor were any WSN proteins from singly infected samples detected. The nature of this material is presently unclear.

VSV G protein has previously been shown to be excluded from fowl plague virus in doubly infected cells (40). Because the HHG protein did not incorporate into WSN virus, it seemed possible that the exclusion of G was entirely due to its cytoplasmic domain. To test this possibility, double infections were repeated with SV40-infected cells expressing G or  $G^{\text{tail-}}$ ; the results are shown in Fig. 5. A very small amount of G was detected in fractions containing influenza virus (panels A and B), but a control experiment showed that this could be accounted for by the adsorption of G protein shed into the medium to the surface of influenza virions. In the control experiment, CV-1 cells were infected separately with the SV40 vector expressing G or with WSN virus and were labeled as in double infections. The media from the two cultures were collected and mixed together for 2 h at 37°C. Virions from this mixed cell culture supernatant were then

centrifuged on gradients as before. The gradients were collected in fractions which were mixed with detergent to dissociate viral proteins and were immunoprecipitated first with anti-WSN antibody (Fig. 5 panel B) and then with anti-G antibody. G protein was detected in gradient fractions containing influenza virus when G was added to virus after harvest (panel C), similar to the results when G was present continuously at the plasma membrane of infected CV-1 cells (panel A). These results show that G protein expressed from SV40 vectors was excluded from influenza virions, just as had been reported previously for double infections with influenza viruses and VSV (40). Because no VSV internal proteins were present in our experiments, this exclusion is due to the inability of G to compete for space in the WSN envelope. Steric hindrance caused by the G cytoplasmic domain does not explain this lack of incorporation, because  $G^{\text{tail-}}$  was equally excluded (panel A).

Fluorograms from immunoprecipitations of gradient fractions containing viruses produced by doubly infected cells expressing recombinant proteins were properly exposed for



TABLE 2. Distribution of proteins in virions and at the cell surface<sup>a</sup>

Protein	Ratio of protein in virus to protein on cell surface	Ratio of expression of recombinant proteins to WSN HA:	
		In virus	On cell surface
A/WSN	0.50	1.0	1.0
Japan HA	0.55	0.4	0.2
HA <sup>tail-</sup>	0.33	0.2	0.2
HHG	0.01	0.002	0.2
HHgC	0.01	0.002	0.2
HgCH <sup>b</sup>	0.08	0.004	0.1
VSV G <sup>tail-b</sup>	0.01	0.001	0.2
VSV G <sup>b</sup>	0.01	0.002	0.2

<sup>a</sup> Values are averages from three independent experiments and are the results of densitometric scanning of fluorograms of immunoprecipitated radioactive proteins. Virus was collected by velocity sedimentation of cell culture medium on sucrose gradients. The surface population of HA proteins was that accessible to trypsin added to culture medium at 4°C. HA proteins inaccessible to trypsin were considered inside cells. The surface population of G protein was the fraction that bound anti-G antibody at the cell surface at 4°C before cell lysis. The remaining protein recovered by a second immunoprecipitation of the same cell lysate was considered to be within the cell.

<sup>b</sup> Values are averages of two experiments.

laser scanning densitometry, and the values for each gradient fraction were plotted in Fig. 6. After double infection, Japan HA was 34% of the total HA in WSN virus. HA<sup>tail-</sup> composed about 22%, and the other proteins were largely excluded. When these data are corrected for differences in the amount of each virus glycoprotein at the cell surface (Table 2), Japan HA was found to be incorporated into the viral envelope as efficiently as the WSN HA was. The percentage of Japan HA in virus was 50 to 55% of the amount of Japan HA on the cell surface (which could include virions not yet released). WSN HA in virions was also 50% of its surface population. HA<sup>tail-</sup> in virions was about 32% of the HA<sup>tail-</sup> on the cell surface, HgCH was about 8%, and HHG and HHgC were less than 3%.

**Neutralization of phenotypically mixed WSN virus.** We have previously shown that the ts61S mutant of WSN virus can be complemented at the nonpermissive temperature by A/Japan HAs (25); thus, WSN virions containing Japan HAs are infectious. If, as our biochemical experiments indicated,

Japan HA and HA<sup>tail-</sup> were incorporated into virus and G and G<sup>tail-</sup> were not, anti-Japan antiserum would be expected to neutralize phenotypically mixed viruses from double infections and anti-G serum would not. Table 3 shows that this was indeed the case. Media collected from single or double infections were preincubated with Japan HA antibody, anti-WSN antibody, both of these sera, or anti-VSV antibody prior to dilution for plaque assays. Anti-WSN serum did not neutralize A/Japan virus but completely neutralized infectivity resulting from all WSN infections except those double infections of WSN and SV40 vectors expressing Japan HA and HA<sup>tail-</sup>. About 1% of the virus produced by these two types of infections was not neutralized by anti-WSN serum (Table 3, lines 3 and 4). All of this residual infectivity was inhibited when virus was incubated with a mixture of anti-Japan and anti-WSN antibody, indicating that this titer was not due to virions in aggregates that had escaped the anti-WSN antibody. The results indicate that pseudotype virions had been produced containing too little WSN HA to be neutralized by anti-WSN antibody. The amounts of anti-Japan serum used were sufficient to completely neutralize titers of A/Japan virus 3 to 4 orders of magnitude higher than those produced by doubly infected CV-1 cells but showed no cross-reactivity with WSN virus (Table 3, line 1). Preincubation with anti-Japan antisera did not significantly reduce the titers of virus produced after infection with WSN alone or by double infections of WSN with SV40-HHG, SV40-HHgC, or SV40-HgCH (Table 3 column 4). However, about half of the infectious units produced from cells doubly infected with either SV40-Japan HA or SV40-HA<sup>tail-</sup> were neutralized (Table 3, lines 3 and 4), indicating the presence of phenotypically mixed virions containing enough Japan HA to be neutralized. Because the biochemical experiments indicated that 34% (wild type) or 22% (HA<sup>tail-</sup>) of the total HA in these virus preparations was Japan HA (Fig. 6), it is likely that at least a portion of the phenotypically mixed virions contained considerable amounts of Japan HA. The 1% of the virus not neutralized by anti-WSN serum (pseudotypes) could not contain that amount of Japan HA.

The neutralization results confirmed that both Japan HA and HA<sup>tail-</sup> were incorporated into the envelopes of phenotypically mixed WSN virus but that very little, if any, of the three chimeric proteins was present. Preincubation with

TABLE 3. Antibody neutralization of phenotypically mixed influenza viruses<sup>a</sup>

Infection <sup>b</sup>	Starting titer (PFU/ml)	Titer (PFU/ml) after treatment with:			
		Anti-WSN antibody	Anti-Japan antibody	Anti-WSN + anti-Japan antibodies	Anti-G antibody
A/Japan	$(1.4 \pm 0.7) \times 10^8$	$(1.4 \pm 0.9) \times 10^8$	0	0	ND <sup>c</sup>
A/WSN	$(5.2 \pm 0.8) \times 10^4$	0	$(5.0 \pm 0.8) \times 10^4$	0	$(5.0 \pm 0.7) \times 10^4$
Japan HA + WSN	$(5.3 \pm 0.6) \times 10^4$	$(3.0 \pm 0.9) \times 10^2$	$(1.8 \pm 0.8) \times 10^4$	0	ND
HA <sup>tail-</sup> + WSN	$(5.2 \pm 0.8) \times 10^4$	$(1.5 \pm 1.0) \times 10^2$	$(2.7 \pm 0.9) \times 10^4$	0	ND
HHG + WSN	$(4.0 \pm 0.5) \times 10^4$	0	$(3.7 \pm 0.3) \times 10^4$	ND	ND
HHgC + WSN	$(3.7 \pm 0.5) \times 10^4$	0	$(4.0 \pm 0.2) \times 10^4$	ND	ND
HgCH + WSN	$5.2 \times 10^4$	0	$5.0 \times 10^4$	ND	ND
G + WSN	$(5.0 \pm 1.4) \times 10^4$	0	ND	ND	$(5.0 \pm 1.0) \times 10^4$
G <sup>tail-</sup> + WSN	$4.0 \times 10^4$	0	ND	ND	$4.0 \times 10^4$

<sup>a</sup> Values are averages  $\pm$  standard deviations of three (Japan HA, HHgC) or four independent experiments. Values without standard deviations are averages of two experiments.

<sup>b</sup> A/Japan virus was prepared in MDCK cells. In each experiment, A/WSN influenza virus was grown for 8 h in CV-1 monkey kidney cells. For double infections, the WSN superinfection was made 32 h after infection with SV40 vectors.

<sup>c</sup> ND, not done.

antisera against VSV had no effect on infectivity of WSN virions produced from cells infected with WSN alone or doubly infected with WSN and vectors expressing G or G<sup>tail-</sup> (Table 3, last column). This observation is consistent with the interpretation that all of the G protein present in gradient fractions containing influenza virions was adsorbed to the outer surface of the virus and was not incorporated into the viral envelope.

## DISCUSSION

It is likely that there are dual mechanisms for excluding foreign proteins from the influenza virus envelope. Two mutants having foreign cytoplasmic sequences, HHG and HHgC, have previously been shown to fold properly into trimeric HA proteins (21), but nevertheless were efficiently excluded from WSN virus. We have previously observed that folding of the external domain is quite sensitive to changes in the transmembrane domain (21); thus, the fact that the external domains of both of these proteins have no detectable differences from wild-type HA suggests that the transmembrane domains were also well folded. The inhibitory effect of the foreign cytoplasmic domains of HHG and HHgC on their assembly into virus probably occurs in the cytoplasm. The highly conserved HA cytoplasmic domain is relatively hydrophobic and may not extend far from the plasma membrane. In contrast, the 11 cytoplasmic amino acids of gC and much longer cytoplasmic sequence of G are more charged and likely to be extended and perhaps are excluded from the viral envelope because they do not fit into a structure formed by the internal proteins of influenza virus. However, because an HA lacking cytoplasmic sequences was efficiently incorporated into influenza virions but a G protein without cytoplasmic sequences was not, steric hindrance of proteins with long or charged cytoplasmic tails is not the only mechanism operating during influenza virus assembly. A second level of selectivity is likely to require binding of the HA transmembrane sequences by an internal virus component. Because of its abundance in the virion, the M1 protein thought to form a protein shell on the cytoplasmic face of the influenza virion envelope (4, 5) is a logical candidate for this binding reaction. The binding of HA to the virus matrix might then crowd out other proteins, including those such as G<sup>tail-</sup> or glycolipid-linked proteins that lack cytoplasmic domains and would not be excluded by a mechanism operating only in the cytoplasm. The other influenza virus envelope proteins, the NA, and the small ion channel M2, must also bind to other viral components, or they too would be excluded from the viral envelope by HA.

We cannot exclude the possibility that the effect of foreign cytoplasmic sequences in preventing incorporation of chimeric HA proteins into influenza virus is on the transmembrane domain. Because we observe that the external domains of HHG and HHgC are properly folded, we feel that it is unlikely that the foreign cytoplasmic sequences change the conformation of the adjacent transmembrane sequences, but we currently have no way of directly measuring this. It may be significant that HgCH, having the HA cytoplasmic sequence and lacking the transmembrane sequences, was incorporated into WSN slightly better than HHG, HHgC, or the two variants of G protein. The sequence required for assembling HA into virions, although primarily within the membrane, may extend into the cytoplasm to some extent.

In cells expressing Japan HA superinfected with WSN virus, one-third to one-half of the virus contains enough Japan HA to be neutralized by anti-Japan serum, but only

1% contains so little WSN HA as to escape neutralization by anti-WSN serum. Under these conditions, Japan HA represents 34% of the total HA found in virions. We have shown previously that pseudotypes of the *ts61S* mutant of WSN containing Japan HA in their envelopes are infectious (25). Thus, if all of the Japan HAs found in the virus preparation were present in viruses essentially lacking the WSN HA, the proportion of pseudotype virus detected by neutralization should have been closer to 34% than to 1%. Therefore, the simplest interpretation of these experiments is that they measure the efficiency with which variants of Japan HA (or VSV G) compete with WSN HA for space within the virus envelope. The experiments do not answer the question of whether HA cytoplasmic sequences are required for nucleating virus budding. The minimum amount of WSN HA that might be present in pseudotypes containing HA<sup>tail-</sup> is not known, and the budding of that virus might require the presence of some WSN HA. Although we have duplicated all of our results by using the *ts61S* mutant of WSN, for the reasons given below, experiments employing influenza viruses having HAs with temperature-sensitive defects in transport to the cell surface cannot be used to answer this question. For the same reasons, we do not know whether the absence of HA might allow other proteins, particularly those lacking cytoplasmic sequences, to be passively incorporated into virions. We have observed that G fails to be incorporated into the *ts61S* influenza virus at the nonpermissive temperature, conditions in which no full-length *ts61S* HA is detected in virions. However, there is a report that at 40°C *ts61S* can form virions that apparently lack virus glycoproteins (27). In this respect the *ts61S* influenza virus mutant resembles the *tsO45* VSV, which also forms "naked" virus particles at 40°C. However, *tsO45* virions formed at the nonpermissive temperature contain large amounts of a fragment of the mutant G protein that includes its cytoplasmic sequences (24), and it is likely that this fragment is responsible for both nucleating virus budding and excluding foreign transmembrane proteins from the viral envelope. The possibility that at the nonpermissive temperature a similar intracellular proteolysis of *ts61S* HA generates a fragment capable of nucleating virion assembly has not been excluded. Investigations of the role of HA in nucleating budding require a system in which HA variants can complement an influenza virus that completely lacks an HA. The recent development of highly efficient recombinant influenza viruses (10) may offer an opportunity for such studies.

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