1986

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JOURNAL OF VIROLOGY, Dec. 1986, p. 994–1001
0022-538X/86/120994-08 $02.00/0

Vol. 60, No. 3

Formation of Influenza Virus Particles Lacking Hemagglutinin on the Viral Envelope

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Received 2 December 1985/Accepted 3 September 1986

We investigated the intracellular block in the transport of hemagglutinin (HA) and the role of HA in virus particle formation by using temperature-sensitive (*ts*) mutants (*ts*134 and *ts*61S) of influenza virus A/WSN/33. We found that at the nonpermissive temperature (39.5°C), the exit of *ts* HA from the rough endoplasmic reticulum to the Golgi complex was blocked and that no additional block was apparent in either the exit from the Golgi complex or post-Golgi complex transport. When MDBK cells were infected with these mutant viruses, they produced noninfectious virus particles at 39.5°C. The efficiency of particle formation at 39.5°C was essentially the same for both wild-type (wt) and *ts* virus-infected cells. When compared with the wt virus produced at either 33 or 39.5°C or the *ts* virus formed at 33°C, these noninfectious virus particles were lighter in density and lacked spikes on the envelope. However, they contained the full complement of genomic RNA as well as all of the structural polypeptides of influenza virus with the exception of HA. In these spikeless particles, HA could not be detected at the limit of 0.2% of the HA present in wt virions. In contrast, neuraminidase appeared to be present in a twofold excess over the amount present in *ts* virus formed at 33°C. These observations suggest that the presence of HA is not an obligatory requirement for the assembly and budding of influenza virus particles from infected cells. The implications of these results and the possible role of other viral proteins in influenza virus morphogenesis are discussed.

Influenza virus is an enveloped virus that contains two glycoproteins, hemagglutinin (HA) and neuraminidase (NA), and matures at the plasma membrane of infected cells by budding (23). The sequence of events leading to the assembly and release of mature influenza virions is poorly understood. Previous results suggested that the viral envelope proteins are first incorporated into the plasma membrane of infected cells and form patches by excluding cellular proteins (6, 11). Subsequently, these regions of modified membrane initiate the formation of an electron-dense layer of matrix (M_1) protein on the cytoplasmic face and apparently provide the affinity for the attachment of viral nucleocapsids, leading to the initiation of the budding process. The first step in this process, i.e., the localization of viral glycoproteins on membrane domains of infected cells, has been proposed to provide the determinants for the site of virus maturation (reviewed in references 9 and 29). In support of this view, it is known that influenza viruses bud exclusively from the apical domain of the plasma membrane in polarized epithelial cells (31). This is the domain to which both HA and NA are transported in influenza virus-infected cells (31) and in cells expressing these glycoproteins from cloned cDNAs (14, 32). Furthermore, of the two glycoproteins, HA is found to be concentrated in discrete regions on the plasma membrane of influenza virus-infected cells, whereas NA is dispersed throughout the membrane (11). It has also been suggested that cells infected with a temperature-sensitive (*ts*) mutant (*ts*61S) of influenza virus which displays a defect in the intracellular transport of HA at the nonpermissive temperature fail to produce virus particles (35). Conversely, virus budding occurs in cells infected with *ts* NA mutants (26), which have recently been shown to be defective in the transport of NA to the cell surface (3a). In addition, it is known that the NA polypeptide is completely absent in influenza type C viruses. The evidence implies that HA is involved in the budding process.

To directly assess the role of HA in virus assembly, we examined two *ts* mutants (*ts*61S and *ts*134) of influenza virus for the maturation and assembly of virus particles at the nonpermissive temperature. These mutants possess a block in the transport of HA to the cell surface at the nonpermissive temperature (28, 30, 35, 36) which is caused by single nucleotide substitutions in the HA gene (S. Nakajima, M. Ueda, K. Nakajima, A. Sugiura, D. J. Brown, A. K. Pattnaik, and D. P. Nayak, Virology, in press). In this communication, we report that noninfectious influenza virus particles are formed at the nonpermissive temperature from cells infected with these mutants and that these noninfectious particles lack HA on the viral envelope. These results suggest that the presence of HA is not an obligatory requirement for the budding and release of influenza viruses.

MATERIALS AND METHODS

Cells and viruses. Madin-Darby bovine kidney (MDBK) cells were obtained from the American Type Culture Collection and maintained in Eagle minimum essential medium containing 10% fetal bovine serum. Wild-type (wt) influenza virus strain A/WSN/33 was plaque purified on MDBK cells, and virus stocks were made from individual plaques as described previously (25). The *ts* mutants (*ts*61S and *ts*134), derived from the WSN strain, were kindly provided by A. Sugiura. These mutants were plaque purified at the permissive temperature (33°C) on MDBK cells, and individual plaques were grown at 33°C as stock viruses. Stock viruses having a 1,000-fold or more reduced infectivity (PFU) titer at the nonpermissive temperature (39.5°C) were used in the experiments. The titrations of infectivity and humagglutinating units were performed as described previously (13).

Radioisotopic labeling of virus-infected cell monolayers. Confluent monolayers of MDBK cells grown on 35- or

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60-mm tissue culture dishes (Falcon) were infected with the wt or *ts* mutants at an input multiplicity of infection of 5 to 10 PFU per cell. Infected cells were incubated at 33 or 39.5°C (depending on the protocol of the experiment) in maintenance medium containing 0.2% bovine serum albumin (25). For labeling proteins with [^{35}S]methionine, the cell monolayers were washed and incubated in methionine-free medium at the appropriate temperature 20 min prior to the addition of the label. Steady-state labeling of viral proteins was performed at 5 h postinfection (p.i.) in methionine-free medium containing 25 μCi of [^{35}S]methionine (1,300 to 1,500 Ci/mmol; Amersham Corp.) per ml. Pulse-labeling (usually for 10 to 15 min) was done similarly, except that the amount of [^{35}S]methionine was increased to 100 μCi/ml. After being labeled, the cell monolayers were washed with cold phosphate-buffered saline (PBS) and processed for immunoprecipitation.

Immunoprecipitation and endo-H digestion. The infected cell monolayer was scraped into cold PBS, pelleted, and lysed in radioimmunoprecipitation assay (RIPA) buffer containing 10 mM Tris hydrochloride (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1 mM phenylmethylsulfonyl fluoride at 0°C. The lysate was clarified by centrifugation at 12,000 × *g* for 10 min at 4°C. The clarified lysate (0.2 to 0.5 ml) was mixed with 3 μl of murine anti-HA (A/PR/8/34) monoclonal antibody (H15-A13-18) (10) and incubated for 2 h at 0°C. One hundred microliters of a protein A-Sepharose slurry (about 5 mg, equilibrated in RIPA buffer) was added, and the mixture was incubated for 30 min at 0°C. The protein A-Sepharose immune complex was washed three times with RIPA buffer, and the immune complex was eluted in RIPA buffer by heating at 95°C for 3 min. The immune complex was concentrated by precipitation with 8 volumes of cold acetone (2 h at −20°C) and suspension of the precipitate in 100 to 200 μl of 0.1% SDS. The desired amount of radioactivity was adjusted to 50 mM sodium acetate (pH 5.5) and treated with endo-β-*N*-acetylglucosaminidase H (endo-H) (50 mU/ml) for 16 to 18 h at 37°C. After digestion, the sample was lyophilized and suspended in 25 to 30 μl of sample preparation buffer containing 2% SDS for electrophretic analysis. The sample not digested with endo-H was treated similarly, except that the enzyme was omitted.

Electrophoretic analysis of proteins. The electrophoresis of proteins was performed in 10% polyacrylamide gels containing SDS (SDS-PAGE) by using the buffer system of Laemmli (18). After electrophoresis, the gel was fixed, dried, and fluorographed (4) with Cronex X-ray film at −80°C.

Preparation of labeled virus. MDBK cell monolayers were infected with wt or *ts* virus and incubated in maintenance medium at either 33 or 39.5°C. At 4 h p.i. the infected cultures were incubated in maintenance medium containing half the usual concentration of unlabeled methionine plus 25 μCi of [^{35}S]methionine per ml for 20 to 24 h. The culture fluids were collected and clarified, and the labeled virus was purified by sucrose density gradient centrifugation (5). The virus particles were pelleted by centrifugation, and the pellets were used for the analysis of proteins by SDS-PAGE. For the immunoprecipitation of HA, the virus samples were disrupted in RIPA buffer and immunoprecipitated with anti-HA monoclonal antibodies.

Immunofluorescence. For cytoplasmic immunofluorescence, infected cell monolayers grown on cover slips were washed with cold PBS and fixed in acetone-methanol (1:1 [vol/vol]) at −20°C. For cell surface immunofluorescence, infected cell monolayers were fixed with 1% *para*-formaldehyde (in 0.072 M cacodylate buffer [pH 7.5] and 0.72% sucrose) for 30 min at 4°C. The fixed cells were stained with anti-HA monoclonal antibodies in a humidified chamber at 37°C for 30 min. Subsequently, the cells were washed thoroughly with PBS and stained with fluorescein-conjugated goat anti-mouse immunoglobulins (Cooper Biomedical, Inc.) at 37°C for 30 min. After being thoroughly washed with PBS, the cover slips were mounted with 90% glycerol in PBS containing 0.1% *para*-phenylenediamine and examined immediately with a transmission fluorescence microscope.

NA activity determination. NA activity associated with the virus preparations was determined by the colorimetric method of Aminoff (2) with fetuin as the substrate. The virus from clarified culture fluids was pelleted through a cushion of 30% glycerol in 10 mM Tris hydrochloride (pH 7.4)–100 mM NaCl, and the pellets were suspended in the same buffer without glycerol.

Immunoblotting. Immunoblotting was used for the quantitation of HA and NA. Appropriate amounts of viral protein from the density gradient-purified virus were applied onto nitrocellulose paper by using the slot-blot apparatus of Schleicher & Schuell, Inc. Subsequently, the paper was blocked overnight at room temperature with 5% bovine serum albumin in buffer A (10 mM Tris hydrochloride [pH 7.4], 0.9% NaCl) and then reacted with either anti-HA or anti-NA monoclonal antibodies for 4 to 5 h at room temperature. After the reaction, the paper was washed at room temperature once in buffer A for 30 min, twice in buffer A containing 0.1% Nonidet P-40 for 30 min each time, and once in buffer A for 30 min. The washed paper was then incubated overnight at room temperature in buffer A containing 0.1 μCi of ^{125}I-protein A per ml and 2% bovine serum albumin. The unbound ^{125}I-protein A was removed by washing, and the filter paper was dried and exposed to X-ray film. To analyze NA, we used ^{125}I-sheep anti-mouse immunoglobulin F(ab')$_2$ instead of ^{125}I-protein A.

RESULTS

Characterization of the HA phenotype in *ts*61S and *ts*134. Although *ts*61S and *ts*134 have been shown to be defective in the transport of HA to the cell surface at 39.5°C, reports as to the location of the block are conflicting (28, 30, 36). We therefore reexamined the intracellular location of the block in the transport of HA. Since HA undergoes various post-translational modifications during its intracellular transport (16, 20), we wanted to analyze the various forms of HA synthesized at both the permissive and nonpermissive temperatures. Accordingly, MDBK cells were infected with wt, *ts*134, or *ts*61S virus and incubated at either 33 or 39.5°C. At 5 h p.i., the infected cells were labeled for 30 min with [^{35}S]methionine at the corresponding temperatures, and HA was immunoprecipitated with monoclonal antibodies and analyzed by SDS-PAGE. Since both *ts*61S and *ts*134 produced similar results, only the data obtained from *ts*61S and wt viruses are shown in Fig. 1. Two different forms of HA (HA and HA_0) were found in wt virus-infected cells at both temperatures (lanes A and C) and in *ts* virus-infected cells at 33°C (lane E). At 39.5°C, the *ts* mutant-infected cells (lane G) contained only the faster-migrating HA_0 and not the slower-migrating mature HA. Pulse-chase experiments demonstrated that HA_0 in *ts*61S virus-infected cells at 39.5°C was not converted to HA after a 2-h chase, whereas more than 75% of HA_0 in wt virus-infected cells at 39.5°C was converted to HA after a 2-h chase. HA_0 was completely endo-H

sensitive and produced HA'_0 (M_r, 64,000; lanes B, D, F, and H), whereas mature HA was only partially endo-H sensitive and produced a slightly faster-migrating form of HA (lanes B, D, F, and H). This partial sensitivity of mature HA was presumably due to the presence of some high-mannose sugars, as has been shown for HA present on influenza virus particles (15). These results confirmed the earlier observations that only the endo-H-sensitive HA_0 was produced in *ts* virus-infected cells at 39°C (36).

For further analysis of the block in the intracellular transport of *ts* HA, cells were grown on cover slips, infected with wt or mutant virus, and incubated at either 33 or 39.5°C. At 6 h p.i., they were processed for either surface or cytoplasmic immunofluorescence. The results (Fig. 2) showed that at 33°C, HA was present on the cell surface of both *ts*61S (panel b) and wt (not shown) virus-infected cells, whereas at 39.5°C, HA was observed on the cell surface of wt (panel a) but not *ts*61S (panel c) virus-infected cells. The cytoplasmic fluorescence of wt virus-infected cells at 33°C (not shown) or 39.5°C (panel d) was also similar to that of *ts*61S virus-infected cells at 33°C (panel e). In contrast, *ts*61S virus-infected cells at 39.5°C showed a weak, reticular cytoplasmic fluorescence (panel f), a characteristic of the rough endoplasmic reticulum (RER) staining. The results obtained with *ts*134 at both temperatures were essentially the same at those obtained with *ts*61S.

The results of the immunofluorescence and endo-H digestion studies indicated that the *ts* block in the transport of HA is at the RER. However, to rule out any additional block at the Golgi complex or between the Golgi complex and the cell surface, we examined whether *ts* HA, when present in the Golgi complex, can be transported to the cell surface at the nonpermissive temperature. The strategy of the experiment was to allow the synthesis and accumulation of the mutant HA in the Golgi complex without permitting it to go to the cell surface and then to monitor its transport to the cell surface at either 33 or 39.5°C by immunofluorescence. It has previously been reported that at 20°C, wt HA is not transported to the cell surface but is accumulated in the Golgi complex (22). We used this low-temperature (20°C) block to allow the accumulation of HA in the Golgi complex. Cells infected with wt or *ts* virus were incubated at 39.5°C for 4 h, shifted down to 20°C for 2 h, and then shifted up to either 33 or 39.5°C for 2 h. Additionally, cycloheximide was added 10 min prior to and during the temperature shift-up to stop further synthesis of HA. The results obtained from wt and *ts*61S virus-infected cells are shown in Fig. 2. At 4 h p.i., cytoplasmic staining showed the characteristic weak, reticular RER fluorescence (data not shown). When maintained at 20°C, the cultures showed HA fluorescence mainly in the perinuclear region (indicated by arrowheads in panels g and h), typical of Golgi complex staining (21). The fluorescence pattern was essentially similar in both wt and *ts* virus-infected cells. When the cultures were shifted up to either 33 or 39.5°C, wt HA (panels i and k) as well as *ts* HA (panels j and l) migrated to the cell surface. The cytoplasmic and cell surface distributions of HA following the release of the Golgi complex block were essentially identical in wt and *ts*61S virus-infected cells at the permissive as well as nonpermissive temperatures. Cells infected with *ts*134 virus exhibited similar results (data not shown). These data indicated that there was no additional block in the post-Golgi complex transport of *ts* HA at 39.5°C.

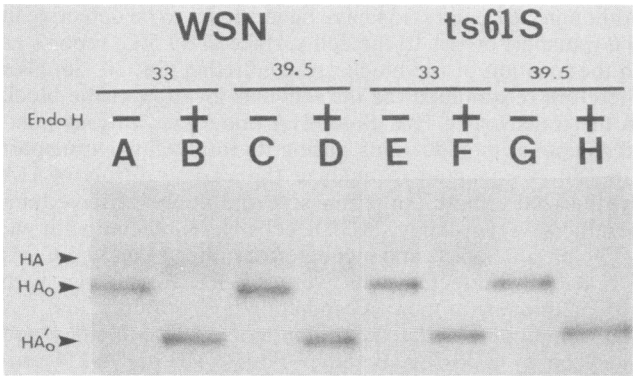

FIG. 1. SDS-PAGE of HA synthesized in cells infected with wt or *ts*61S virus. Confluent monolayers of MDBK cells in 60-mm petri dishes were infected with wt (lanes A to D) or *ts*61S (lanes E to H) virus and incubated at 33°C (lanes A, B, E, and F) or 39.5°C (lanes C, D, G, and H). At 5 h p.i., the infected cells were labeled with [^{35}S]methionine for 30 min in methionine-free medium at the corresponding temperatures. Labeled HA was immunoprecipitated from cell lysates with monoclonal antibodies as described in Materials and Methods and analyzed by SDS-PAGE before (−) or after (+) digestion with endo-H. HA refers to the fully glycosylated uncleaved form of HA, HA_0 refers to the form present in the RER, and HA'_0 refers to the form of HA_0 left after endo-H digestion.

Formation of virus particles in *ts* virus-infected cells at the nonpermissive temperature. Despite the absence of hemagglutinating activity, the culture fluids of the *ts* virus-infected cells incubated at 39.5°C for 24 h contained a significant level of NA activity. Pronounced cytopathic effects were also observed in *ts* virus-infected cells at the nonpermissive temperature. These observations prompted us to investigate whether virus particles lacking HA were being formed and released into the culture fluids at 39.5°C. Accordingly, MDBK cells were infected with wt, *ts*134, or *ts*61S virus at both 33 and 39.5°C. At 24 to 26 h p.i., the culture fluids were clarified and analyzed by equilibrium sucrose density gradient (20 to 65%) centrifugation. Viruslike bands were seen in all gradients, although the band obtained from the *ts* virus-infected cells at 39.5°C had a slightly lower density (1.18 g/ml) than those obtained from the wt virus-infected cells at either temperature or the *ts* virus-infected cells at 33°C (1.20 g/ml). In addition, the supernatant fluid from *ts* virus-infected cells at 39.5°C produced a more diffuse band.

To analyze the nature of the particles produced from *ts* virus-infected cells at 39.5°C, we collected the bands from sucrose gradients and directly examined them by negative staining with a transmission electron microscrope (Fig. 3). The virus particles produced from wt virus-infected cells at 33 or 39.5°C (panels A and B, respectively) and from *ts*134 or *ts*61S virus-infected cells at 33°C (panels C and E, respectively) were seen to possess obvious spikes on the viral envelope. However, the particles produced at 39.5°C from *ts*134 or *ts*61S virus-infected cells were different in morphology, resembling spikeless virus particles (panels D and F, respectively). These particles appeared to be more fragile, and many of them were broken, perhaps explaining the diffuse nature of the bands observed in sucrose gradients. The reduced density of these particles could also be consistent with the absence of HA on the envelope.

To quantitate the relative amount of spikeless virus particles produced from *ts* virus-infected cells at 39.5°C, we labeled cells infected with wt or *ts* viruses at both temperatures with [^{35}S]methionine and measured the incorporation of the label into purified virus particles. Since the electrophoretic analysis of the labeled proteins indicated that the gradient-purified virus preparations contained predomi-

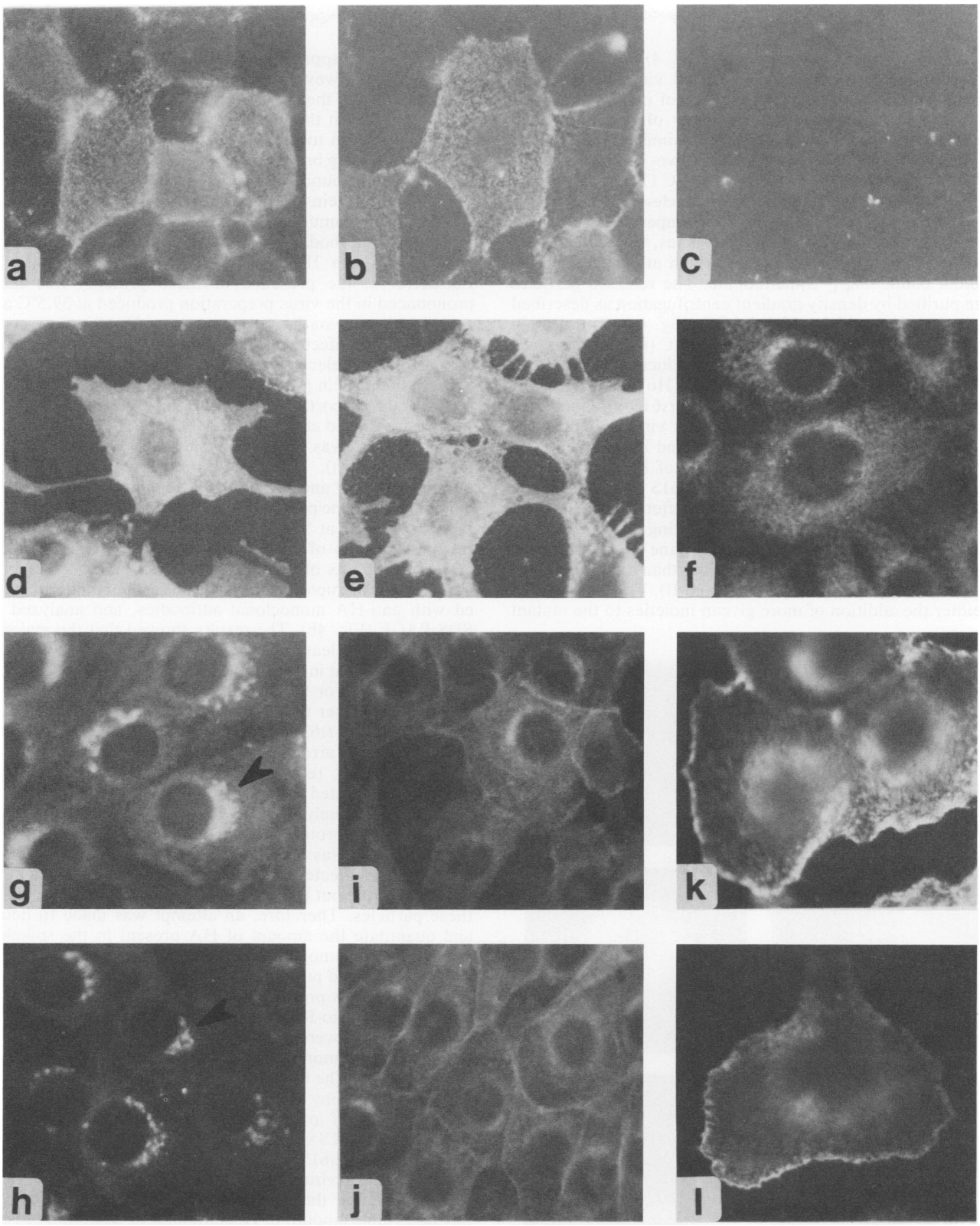

FIG. 2. Cell surface and cytoplasmic distributions of HA in cells infected with wt or *ts*61S virus. Panels a to f show the cell surface (panels a, b, and c) and cytoplasmic (panels d, e, and f) immunofluorescence patterns of MDBK cells infected with wt or *ts*61S virus and incubated at either 33 or 39.5°C for 6 h; panels a and d show wt virus-infected cells at 39.5°C, panels b and e show *ts*61S virus-infected cells at 33°C, panels c and f show *ts*61S virus-infected cells at 39.5°C. Panels g to l show the cytoplasmic and cell surface distributions of HA following the Golgi complex block at 20°C and the subsequent release of the block at either 33 or 39.5°C. Infected cells were incubated at 39.5°C for 4 h, followed by a shift down to 20°C for 2 h to block HA in the Golgi complex; cytoplasmic staining of wt and *ts*61S virus-infected cells is shown in panels g and h, respectively. Subsequently, the Golgi complex block was released by a shift up to either 33 or 39.5°C; cytoplasmic staining of wt and *ts*61S virus-infected cells at 33°C is shown in panels i and j, respectively; cell surface staining of wt and *ts*61S virus-infected cells at 39.5°C is shown in panels k and l, respectively. The arrowheads in panels g and h show granular staining, a characteristic of the Golgi apparatus.

nantly virus-specific polypeptides (Fig. 4), trichloroacetic acid-precipitable counts in the purified virus preparations were taken as an index of the number of particles formed. The results showed that the efficiency of budding of the spikeless virus particles at 39.5°C was similar to that of the wt particles at 39.5°C and was about five- to sixfold lower than that of the wt or *ts* particles at 33°C (Table 1).

Analysis of proteins from purified spikeless particles grown at the permissive and nonpermissive temperatures. To analyze the proteins of the spikeless particles, we infected cells with wt or *ts* virus and incubated them at 33 or 39.5°C in medium containing [^{35}S]methionine. The released particles were purified by density gradient centrifugation as described above and analyzed by SDS-PAGE (Fig. 4a). The protein profile of *ts*61S particles produced at 33°C (lane C) appeared to be identical to that of wt particles produced at either 33 or 39.5°C (lanes A and B, respectively). However, HA was absent in the particles produced from *ts*61S virus-infected cells at 39.5°C (lane D). All of the other viral proteins were present in proportionate amounts. PA and PB2, which were often masked because of the presence of HA (lanes A, B, and C), became clearly visible in the *ts*61S particles lacking HA (lane D) or in the wt particles after treatment with trypsin (data not shown). It is worth noting that at 33°C, the HA present in the mutant viruses (lane C) displayed a slightly slower electrophoretic mobility than did the wt virus HA at either temperature (lanes A and B). This may be due to either the addition of more glycan moieties to the mutant HA at the Golgi apparatus or a conformational change in the mutant HA. However, the former possibility seems more likely because of the slower rate of intracellular transport of the mutant HA at the permissive temperature (unpublished data). In addition to the known viral proteins, a number of proteins migrating between the NP, HA_1 and M_1, HA_2 bands (Fig. 4a) were found to be present in these virus preparations. These proteins appeared to be of host cell origin. They could not be immunoprecipitated by either polyclonal or monoclonal antibodies or by antibodies made against the denatured fusion HA expressed in *Escherichia coli* (8). Furthermore, the presence of these proteins was more pronounced in the virus preparation produced at 39.5°C and varied from preparation to preparation. Finally, they comigrated with uninfected cell proteins, as observed by two-dimensional gel electrophoretic analysis (data not shown).

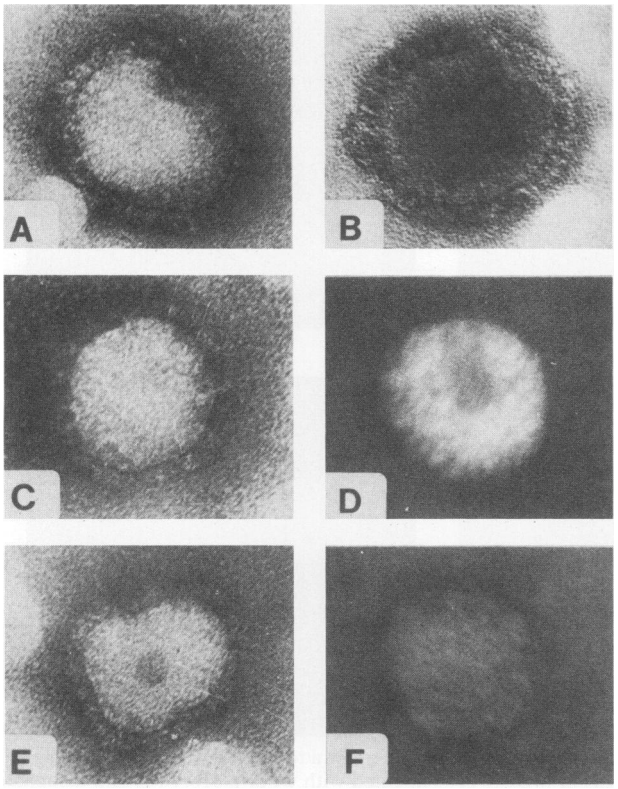

FIG. 3. Negatively stained electron micrographs of virus particles formed at 33 (panels A, C, and E) or 39.5°C (panels B, D, and F). wt or *ts* viruses were grown at either 33 or 39.5°C, purified by successfive sucrose density gradients, and directly visualized under an electron microscope. wt virus is shown in panels A and B, *ts*134 virus is shown in panels C and D, and *ts*61S virus is shown in panels E and F. ×225,000.

From the protein profile shown in Fig. 4a, it appeared that a significant proportion of HA was not cleaved, even in the wt virus produced at either 33 or 39.5°C. The majority of wt HA, however, was cleaved in particles produced at 37°C (data not shown). Since HA_1 and HA_2 migrated in the proximity of NP and M_1 proteins, respectively, these data did not rule out the presence of HA in the cleaved form in the *ts* virus formed at 39.5°C. Therefore, for determining the relative amounts of HA_1 and HA_2 in the wt and *ts* virus particles, aliquots of the labeled virus preparations used in Fig. 4a were disrupted with RIPA buffer, immunoprecipitated with anti-HA monoclonal antibodies, and analyzed by SDS-PAGE (Fig. 4b). The results showed that the majority of HA was not cleaved and that small amounts of HA_1 and HA_2 were present in the wt virus at 33 or 39.5°C (lanes A and B, respectively) or the *ts*61S virus at 33°C (lane C). However, HA in either the cleaved or the uncleaved form was absent from the *ts*61S virus at 39.5°C (lane D). The bands indicated by the arrows above HA_1 and below HA_2 are NP and M_1 proteins, respectively, which were nonspecifically immunoprecipitated.

Quantitative analysis of HA and NA. The electrophoretic analysis of the proteins from purified virions (Fig. 4) indicated that HA was absent in the virions formed at 39.5°C from *ts* virus-infected cells. However, these data could not completely rule out the presence of a small amount of HA in these particles. Therefore, an attempt was made to detect and quantitate the amount of HA present in the spikeless virions by immunoblotting. Accordingly, unlabeled virus was prepared and purified. The total viral protein present in the purified virus preparations was quantitated by a protein assay method (Bio-Rad Laboratories). Appropriate amounts of viral protein were blotted onto nitrocellulose paper and reacted with a monoclonal antibody to HA, followed by ^{125}I-protein A. The bound ^{125}I-protein A was then detected by autoradiography (Fig. 5A). HA could be detected in as little as 7.8 ng of total viral protein in the wt virus at 33 or 39.5°C or the *ts*61S virus at 33°C. However, HA could not be detected in the *ts*61S virus formed at 39.5°C even when the total amount of viral protein was increased to 5 μg. These results indicated that the spikeless virions did not contain any detectable amount of HA at a limit of detection of 0.2% of the HA present in wt virions formed at 33 or 39.5°C or *ts*61S virions formed at 33°C.

A similar analysis was performed to quantitate the amount of NA present in the spikeless virions. Various amounts of total viral protein from purified virions were applied to nitrocellulose paper, reacted with a monoclonal antibody to NA, followed by ^{125}I-sheep anti-mouse immunoglobulin $F(ab')_2$, and autoradiographed. The NA protein was quanti-

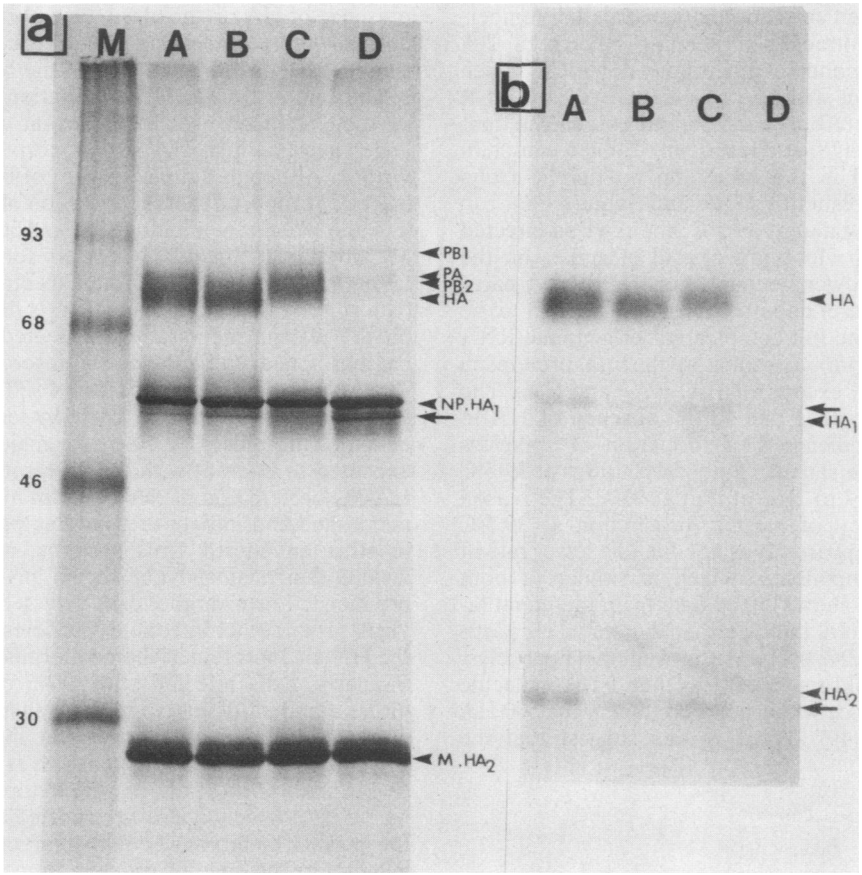

FIG. 4. Electrophoretic analysis of polypeptides present in purified virus particles grown at 33 or 39.5°C. [^{35}S]methionine-labeled purified virus particles were analyzed by SDS-PAGE and fluorographed. Lanes: A and C, wt and *ts*61S viruses grown at 33°C; B and D, wt and *ts*61S viruses grown at 39.5°C. ^{14}C-labeled protein markers (lane M) and their molecular weights (in thousands) are shown. The identity of the protein indicated by the arrow is not known, but it is likely to be a posttranslationally modified form of NP (1, 38). M at the lower right-hand corner is M_1. (b) SDS-PAGE of immunoprecipitated HA from purified virus particles grown at 33 or 39.5°C. [^{35}S]methionine-labeled viruses were disrupted with RIPA buffer, and HA was immunoprecipitated with monoclonal antibodies. Immunoprecipitates were analyzed by SDS-PAGE and detected by fluorography. Lanes: A, wt virus at 33°C; B, wt virus at 39.5°C; C, *ts*61S virus at 33°C; D, *ts*61S virus at 39.5°C. The bands above HA_1 and below HA_2 (indicated by arrows) are NP and M_1, respectively.

tated by densitometric tracing of the autoradiogram and by direct counting of the radioactivity in the bands. The results (Fig. 5B) showed that the amounts of NA present in the wt particles formed at either 33 or 39.5°C and the *ts* particles formed at 33°C were essentially the same. However, the amount of NA in the *ts*61S particles formed at 39.5°C was consistently twofold higher than that in the *ts*61S particles formed at 33°C.

DISCUSSION

The results reported here confirm previous reports (28, 30, 35, 36) that the HA synthesized at the nonpermissive temperature in both *ts*61S and *ts*134 virus-infected cells is transport defective. Our data also indicate that *ts* HA was blocked in the RER without any additional post-Golgi complex block. This conclusion is based on the following observations. (i) The mutant HA synthesized at 39.5°C was completely sensitive to endo-H, as expected for a glycoprotein present in the RER (34, 37). (ii) Indirect immunofluorescence of cells infected with mutant viruses at 39.5°C showed a reticular cytoplasmic fluorescence, as expected for proteins present in the RER (21). Neither cell surface staining nor Golgi complex staining was observed. (iii) When *ts* HA was accumulated at 20°C in the Golgi apparatus, it could be transported to the cell surface upon shifting to the nonpermissive temperature, i.e., when the block between the RER and the Golgi complex was overcome, there was no additional block in the transport of HA to the plasma membrane. In addition, the cytoplasmic immunofluores-

TABLE 1. Efficiency of budding of spikeless virions[a]

Virus	Temp (°C)	[^{35}S]methionine cpm (10^6) in purified virus bands[b]	% Efficiency of budding[c]
wt	33	2.95	100
	39.5	0.56	18
*ts*61S	33	3.02	102
	39.5	0.56	18

[a] Equal numbers (3×10^7) of cells were infected with wt or *ts* virus at a multiplicity of infection of 3 and incubated at the appropriate temperatures. At 24 h p.i., viruses from clarified culture supernatants were purified by density gradient centrifugation, and the trichloroacetic acid-precipitable counts in each virus band were determined.

[b] Data from one experiment.

[c] Average from three separate experiments.

cence of *ts* virus-infected cells at the nonpermissive temperature resembled the intracellular staining pattern of HA obtained by using the monoclonal antibody Y8-10C2, which has been shown to react with HA associated with the RER only (3). Our data, therefore, confirm and extend the conclusion of Rindler et al. (28) and Rodriguez-Boulan et al. (30) that *ts* HA is blocked in the RER and not in the Golgi apparatus, as was suggested by Ueda and Sugiura (36).

Our results also show that at 39.5°C, the *ts* virus-infected cells produce particles which are devoid of spikes on the envelope and have a lower bouyant density than wt particles. These particles are noninfectious and appear to be fragile. They contain the full complement of genomic RNA segments (data not shown), as well as all the viral proteins in proportionate amounts, except for HA, HA_1, and HA_2. The lack of spikes, therefore, is due to the absence of HA in these particles. The efficiency of formation of spikeless virions is essentially the same as that of wt virions at 39.5°C and is approximately 17 to 20% of that at 33°C. The reason for the lower efficiency of particle production at 39.5°C remains unknown but apparently is not due to a lower rate of synthesis of viral components, which is similar at both temperatures (data not shown). The data from the quantitative measurements of HA indicate that the spikeless particles contain less than 0.2% of the HA present in wt particles. Theoretically, this would represent less than 1 HA molecule per spikeless virion, since wt particles contain about 500 HA molecules per virion (19). These results suggest that the presence of HA on the plasma membrane of infected cells is not obligatory for budding and that its absence does not severely impair the efficiency of the budding process.

The demonstration of the formation of virions lacking HA on the envelope raises an important question as to the role and requirement of HA in the morphogenesis of influenza virions. Although the mechanism of budding is not clear, it has been proposed that the insertion of functional HA spikes into the plasma membrane (35) and its interaction with the M_1 protein (20) might be necessary for budding. This view is favored, as biochemical (11) and electron-microscropic (6, 7) data suggest that HA, unlike NA, is distributed into patches on the plasma membrane of infected cells and that these patches appear to be the site of insertion of the M_1 protein. The M_1 protein, which possesses self-polymerizing properties, could cross-link the carboxy-terminal domain of the envelope proteins. HA, being the major envelope protein, is expected to interact with the M_1 protein. The formation of HA-M_1 protein complexes could induce a curvature on the membrane and initiate the budding process (12). However, in other enveloped virus systems, noninfectious particles lacking their major glycoproteins have been reported to be produced. For example, cells infected with *ts*271 of Sendai virus produce noninfectious particles which are devoid of the HN glycoprotein at the nonpermissive temperature (27). Similarly, cells infected with *ts*045 of vesicular stomatitis virus, a mutant defective in the transport of G protein (17), produce noninfectious particles at the nonpermissive temperature (33). In cells infected with *ts* influenza virus defective in the transport of HA, the cytoplasmic carboxy terminus of NA, which is also transported to the same domain of the plasma membrane, could interact with the M_1 protein and initiate the budding process. Since NA is a minor protein component of the viral envelope, present at about 100 molecules per virion (19), little is known about its role in budding. Quantitative measurements show that when compared with *ts* virions produced at the permissive temperature, spikeless virions contain twice as much NA. Recent immunoelectron-microscopic studies on purified wt virus particles have shown that NA is localized in discrete areas on the viral envelope (24) and may, therefore, form patches and trigger budding in the absence of HA. Whether NA is critically required in the budding process of the spikeless virions remains to be seen.

Although unlikely, it is possible that degraded forms of viral HA, particularly portions of the carboxy-terminal anchor and cytoplasmic tail region, may be present on the viral envelope and may be responsible for budding. Attempts to demonstrate the presence of degraded forms of HA in noninfectious virus particles by immunoprecipitation with a variety of antisera yielded negative results but did not completely rule out the presence of minor amounts of degraded forms of HA. However, in the absence of any increased instability of the mutant HA at 39.5°C (unpublished data), this seems to be an unlikely possibility.

The data presented in this paper indicate that the presence of the major viral glycoprotein, HA, on the plasma membrane is not essential for the budding of influenza virions. However, whether the presence of one of the two glycoproteins (i.e., either HA or NA) is critically required for budding remains to be determined. Analysis of the particle formation in cells infected with double mutants possessing defects in the cell surface transport of both HA and NA at the nonpermissive temperature may resolve this issue and provide more definitive answers to questions relating to the role of viral glycoproteins in the budding process.

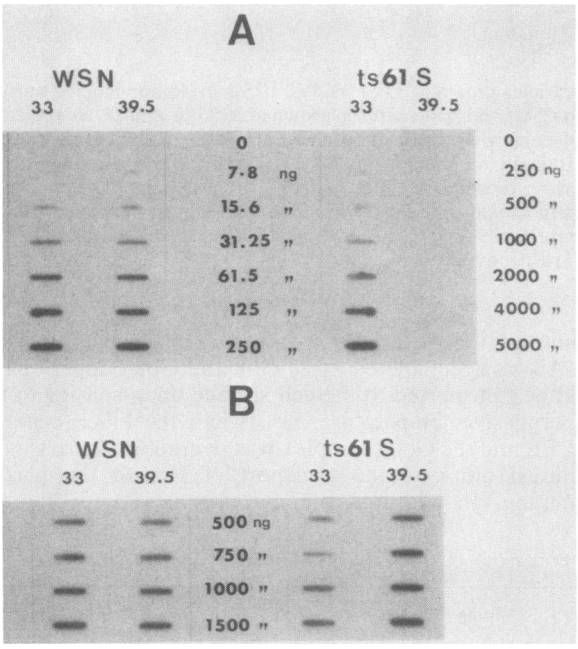

FIG. 5. (A) Quantitative analysis of HA. Various amounts of unlabeled viral proteins (7.8 to 250 ng of WSN (wt) at 33 or 39.5°C and *ts*61S at 33°C or 250 to 5,000 ng of *ts*61S at 39.5°C) were applied onto nitrocellulose paper through a slot-blot apparatus and reacted with a monoclonal antibody to HA, followed by ^{125}I-protein A. The bound ^{125}I-protein A was detected by autoradiography. The limit of detection was 7.8/5,000, or approximately 0.2% of the HA present in wt virus. (B) Quantitative analysis of NA. Various amounts of unlabeled viral proteins (as indicated) were applied onto nitrocellulose paper and reacted with a monoclonal antibody to NA, followed by ^{125}I-sheep anti-mouse immunoglobulin $F(ab')_2$. The bound immunoglobulin was then detected by autoradiography.

ACKNOWLEDGMENTS

We thank Zane Price for his assistance with electron microscopy and Nghia Tran for the preparation of the manuscript. Monoclonal antibodies were a gift from W. Gerhard.

This work was supported by Public Health Service grant R01 AI 16348 from the National Institute of Allergy and Infectious Diseases. D.J.B. was supported by award 5T32CA09030 from the National Cancer Institute.

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