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Surface Expression of Influenza Virus Neuraminidase, an Amino-Terminally Anchored Viral Membrane Glycoprotein, in Polarized Epithelial Cells

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We have investigated the site of surface expression of the neuraminidase (NA) glycoprotein of influenza A virus, which, in contrast to the hemagglutinin, is bound to membranes by hydrophobic residues near the NH_2-terminus. Madin-Darby canine kidney or primary African green monkey kidney cells infected with influenza A/WSN/33 virus and subsequently labeled with monoclonal antibody to the NA and then with a colloidal gold- or ferritin-conjugated second antibody exhibited specific labeling of apical surfaces. Using simian virus 40 late expression vectors, we also studied the surface expression of the complete NA gene (SNC) and a truncated NA gene (SN10) in either primary or a polarized continuous line (MA104) of African green monkey kidney cells. The polypeptides encoded by the cloned NA cDNAs were expressed on the surface of both cell types. Analysis of [^{3}H]mannose-labeled polypeptides from recombinant virus-infected MA104 cells showed that the products of cloned NA cDNA comigrated with glycosylated NA from influenza virus-infected cells. Both the complete and the truncated glycoproteins were found to be preferentially expressed on apical plasma membranes, as detected by immunogold labeling. These results indicate that (i) the NA polypeptide contains structural features capable of directing the transport of the protein to apical cell surfaces and (ii) the first 10 amino-terminal residues of the NA polypeptide are not involved in this process.

In polarized epithelial cells, the maturation sites of enveloped viruses that form by budding at cell surfaces are restricted to particular membrane domains (1, 21, 27). Influenza A, influenza C, and two paramyxoviruses have been found to selectively bud from the apical surface of polarized cells (12, 21, 25), whereas the basolateral surface is the preferred site of maturation for vesicular stomatitis virus (VSV) (21) and several retroviruses (27). The maturation sites reflect the sites of insertion of the respective viral glycoproteins (11, 20). It has been proposed that the cellular transport machinery recognizes a structural feature in the viral polypeptides, thus determining the ultimate insertion sites for these glycoproteins (25, 27). Evidence has been obtained that glycosylation is not required for polarized surface expression of viral glycoproteins (3, 11, 26). Roth et al. (25) reported that expression of the cloned hemagglutinin (HA) gene of influenza A virus, in the absence of other influenza components, occurs almost exclusively on the apical surfaces of primary African green monkey kidney (AGMK) epithelial cells, indicating that the cellular machinery involved in directional transport to apical surfaces recognizes structural features of the HA molecule.

In this report, we have investigated the site of surface expression of the other envelope glycoprotein of influenza A virus, the neuraminidase (NA). The mature NA glycoprotein is anchored in the virion envelope via its NH_2 terminus, in contrast to the HA glycoprotein, which is anchored by the COOH terminus (5, 9, 13). Because of this difference in membrane orientation, it was of interest to investigate the site of surface expression of NA on influenza virus-infected polarized cells. In addition, we compared the site of surface expression of NA by using a complete and a truncated NA gene that were cloned (8) into the late region of simian virus 40 (SV40).

MATERIALS AND METHODS

Cells and viruses. The MA104 line of AGMK cells was obtained from M. Roth. Primary AGMK cells were purchased from MA Bioproducts, Walkersville, Md. These cells were maintained in Dulbecco modified minimum essential medium (MEM) supplemented with 10% fetal calf serum. Madin-Darby canine kidney (MDCK) cells were grown as described previously (23).

Recombinant SV40 vectors expressing either the complete NA gene (SNC) or an NA gene lacking the sequence encoding the first 10 residues (SN10) and the helper virus (SVSal.32) have been described previously (8). Stocks of the A/WSN/33 (H1N1) strain of influenza virus were prepared in Madin-Darby bovine kidney (MDBK) cells (7). Stocks of VSV, Indiana serotype, were prepared in BHK-21 cells (23).

Antibodies and immunospecific labeling. Anti-NA monoclonal antibody was obtained from W. Gerhard, Wistar Institute, Philadelphia, Pa. The production of anti-HA monoclonal antibody and rabbit antiserum against isolated VSV G protein has been described previously (2, 24). Anti-influenza virus antiserum was prepared in rabbits as described previously (23). Fluorescein-conjugated goat anti-rabbit immunoglobulin G (IgG) was purchased from Miles Laboratories, Elkhart, Ind. Ferritin-conjugated anti-mouse IgG and anti-rabbit IgG were purchased from Cappel Laboratories, Cochranville, Pa. Colloidal gold (5-nm particle size)-conjugated goat anti-mouse immunoglobulin was purchased from Janssen Pharmaceutica, Piscataway, N.J.

For indirect immunofluorescent staining, cells were grown on glass cover slips (12-mm circle) in 24-well tissue culture cluster plates (Costar, Cambridge, Mass.) and infected with either influenza virus (multiplicity of infection, 10) or undi-

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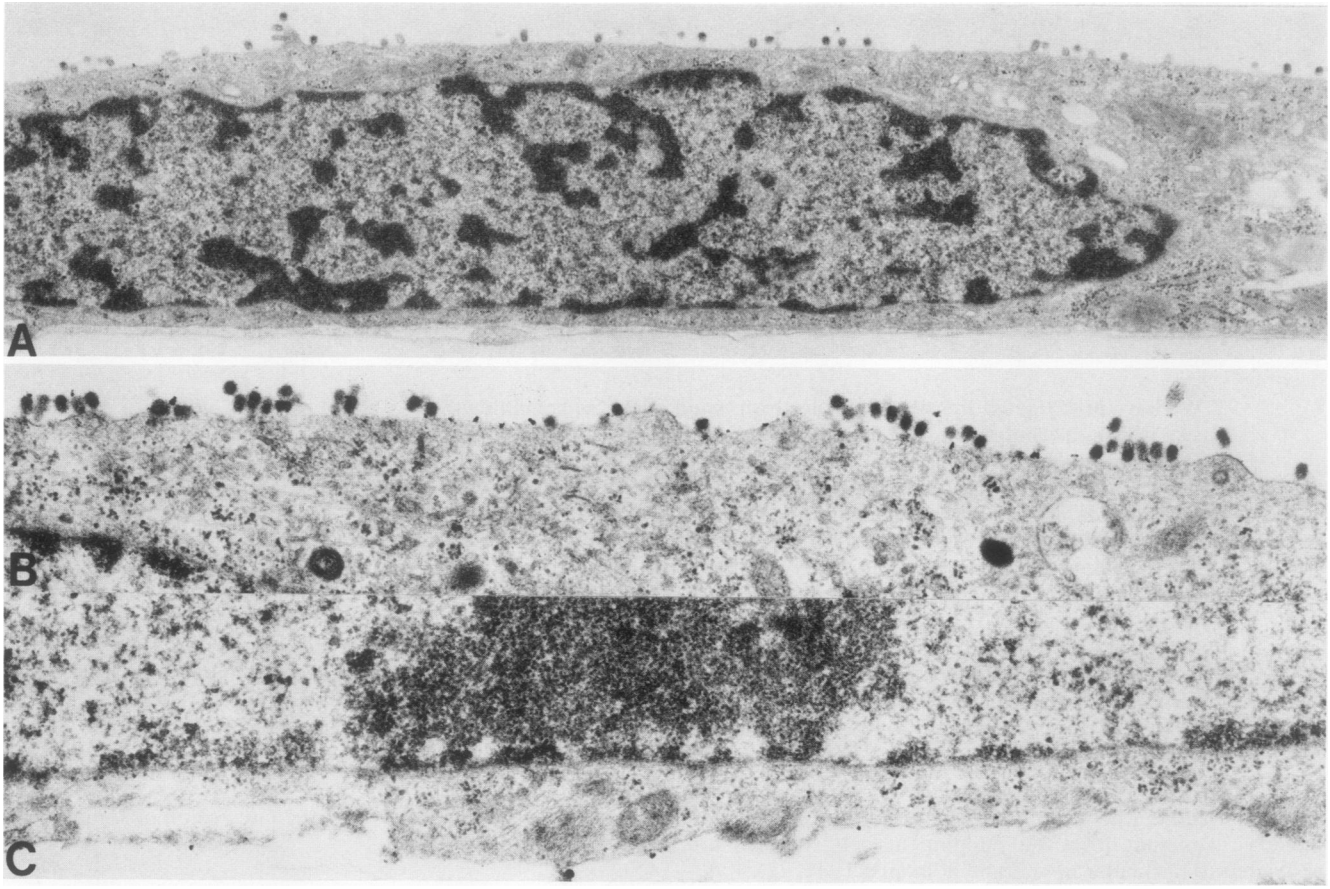


FIG. 1. Polarity of influenza virus maturation in MA104 (A) and AGMK (B and C) cells. (A) Influenza virions are shown budding from the apical surface of an MA104 cell 8 h postinfection. The basal surface of the same cell is free of virions (×14,000). (B and C) Micropore filters were incubated overnight at 37°C in growth medium. AGMK cells were seeded on these filters, and at confluency they were infected with influenza virus. At 8 h postinfection the filters were rinsed, fixed in glutaraldehyde, and processed for electron microscopy. (B) Influenza virions budding from the apical membrane of an AGMK cell (×25,000). (C) Basal surface of same cell, showing no virions (×25,000).

luted recombinant virus stock. At appropriate times postinfection, the cells were incubated with rabbit antiserum to influenza virus (1:20 in phosphate-buffered saline containing 0.1% bovine serum albumin [PBS-BSA]), followed by incubation with fluorescein-conjugated anti-rabbit IgG (1:15). The cells were washed, mounted, observed for fluorescence, and photographed with a Nikon Optiphot microscope equipped with a modified B2 cube.

For immunoelectron microscopy, cells were grown on presoaked mixed ester cellulose-nitrate-acetate filters (type SSWP, 3-μm pore size; Millipore Corp., Bedford, Mass.) placed in the wells of a 24-well plate. These cells were infected with influenza virus or VSV at a multiplicity of 10 PFU per cell or with undiluted recombinant virus stock. At appropriate times postinfection, the filters were washed extensively in PBS and soaked in Eagle MEM containing 5% newborn calf serum for 30 min on ice. To block the nonspecific binding sites on the filter, the filters were incubated on ice with Eagle MEM containing 1% BSA and unconjugated ferritin (100 μg/ml) for 20 min. The filters were then washed and incubated in the appropriate primary antibody (1:20) for 30 min at 20°C, followed by extensive washing with PBS-BSA, and then incubated with a colloidal gold conjugate (1:5) or a ferritin conjugate (1:2) for 30 min at 20°C. The filters were washed extensively, fixed with 1% glutaraldehyde in PBS, and embedded in epoxy resin for electron microscopy. Specimens were examined with a Philips EM301 microscope.

Radiolabeling of infected cells and immunoprecipitation. At 4 h or at 30 to 36 h postinfection, influenza virus-infected and recombinant virus-infected cells, respectively, grown in 35-mm dishes (NUNC, Denmark) were labeled with D-[2-^{3}H]mannose (13.4 Ci/mmol; Amersham Corp., Amersham, U.K.) (50 μCi/dish) in 0.8 ml of Eagle MEM. The cells were incubated in the isotope-containing medium for the final 4 h (influenza virus) or 12 h (recombinant virus) of a single-cycle infection. At the end of the incubation period, the cells were rinsed five times with cold PBS and then lysed with cold cell dissociation buffer (0.01 M Tris-hydrochloride, pH 8.0, 0.25 M NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate). Radiolabeled polypeptides were immunoprecipitated with formalinized *Staphylococcus aureus* cells that had been incubated with rabbit antiserum to influenza virus for 1 h at 20°C. The precipitates were washed once with lysis buffer (0.05 M Tris-hydrochloride, pH 7.4, 0.15 M NaCl, 0.02 M EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodceyl sulfate [SDS]) and twice with cell dissociation buffer. The final precipitates were suspended in sample-reducing buffer (0.067 M Tris-hydrochloride, pH 6.7, 6.3% glycerol, 1% SDS, 1% B-mercaptoethanol, 0.006% bromo-

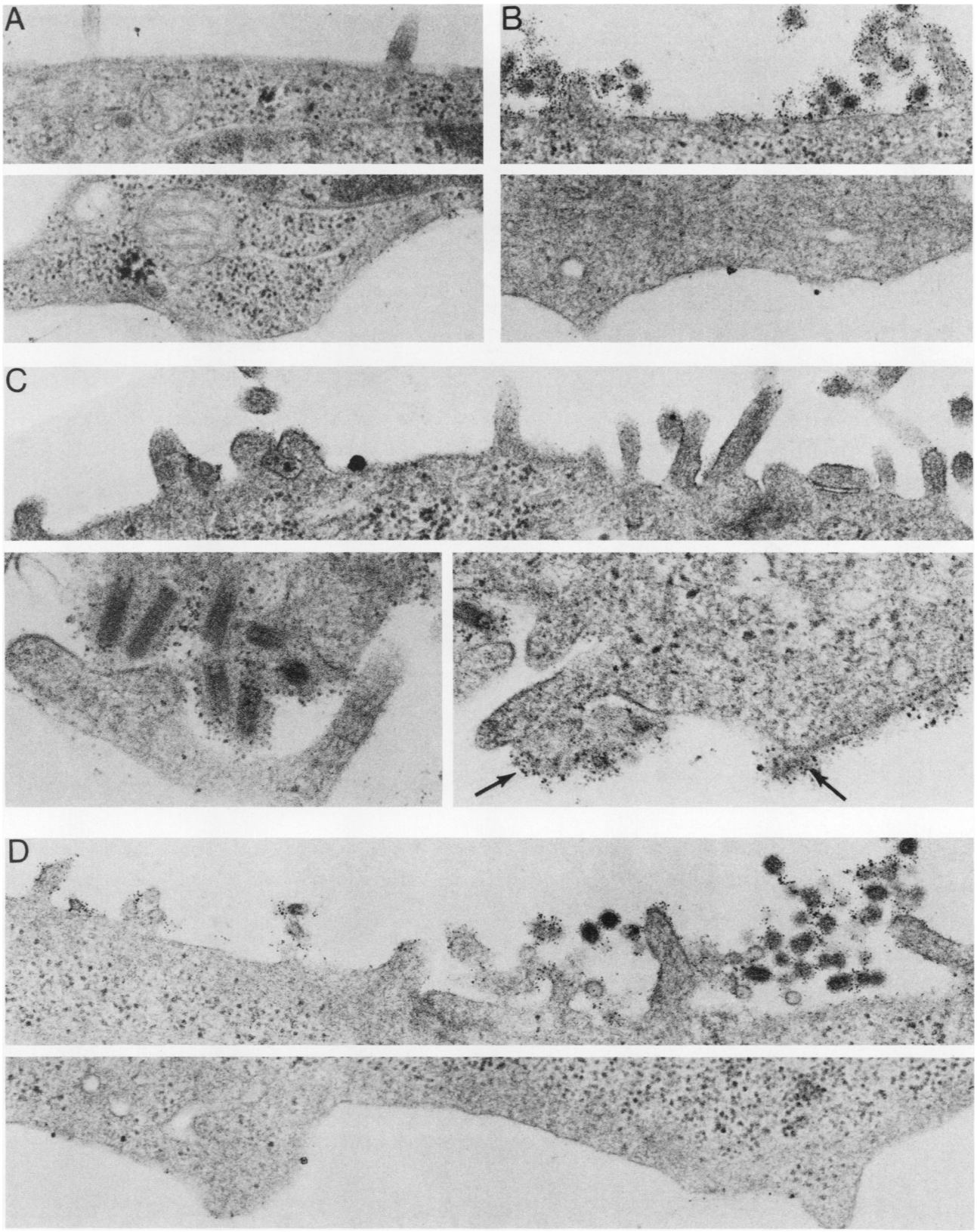
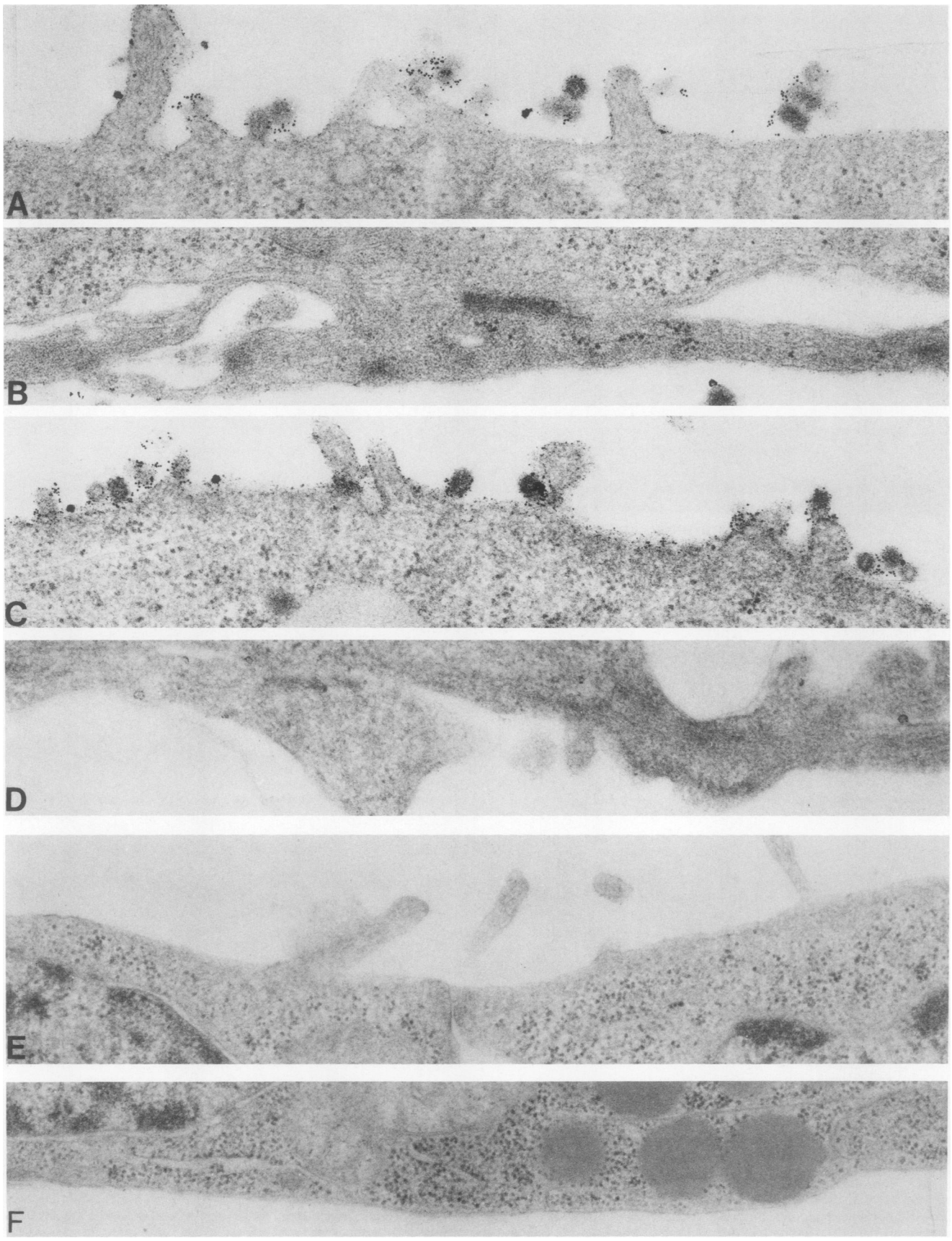


FIG. 2. Polarity of insertion of influenza A virus NA in MDCK cells. Mock infected (A), VSV-infected (C), or influenza virus-infected (B and D) MDCK cells grown on micropore filters were stained with anti-VSV G (A and C), anti-HA (B), or anti-NA (D) and then with ferritin-conjugated second antibody, as described in the text. Panels A, B, and D show apical (top) and basolateral (bottom) aspects of the same cell. Panel C shows the apical surface of an infected cell and the basolateral surfaces of two cells from the same infected monolayer. Arrows indicate the labeling of the basolateral cell surface with ferritin. Magnifications: (A) ×45,000, (B) ×47,000, (C) apical surface, ×54,000, basolateral surface, ×80,000 (left) or ×63,000 (right); (D) ×48,000.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phenol blue), boiled, and analyzed on an SDS–10% polyacrylamide gel (14). The protein bands were visualized by standard fluorography techniques.

RESULTS

Polarized maturation of influenza virus A/WSN/33 in MA104 cells. Because the cDNA of influenza virus NA had been inserted into an SV40 expression vector, we examined NA expression in cells that are permissive for infection by SV40. Both the MA104 line and the primary AGMK cells are permissive for SV40 infection as well as infection by influenza virus and VSV. Roth et al. (25) previously determined that 88% of the influenza virions observed in virus-infected AGMK cells were associated with apical membranes. To determine the site of virus maturation in the continuous MA104 cell line, cells were infected with influenza virus, incubated for 5 to 6 h, and embedded for electron microscopy (Fig. 1A). The majority of the cells observed were connected by desmosomes and tight junctions (not shown) and displayed influenza virions exclusively on apical membranes. Of 540 virions observed, 90% were found budding from or associated with the apical surfaces of the cells. These results are similar to those determined for the polarized MDCK line of epithelial cells in which 92 and 99% of the influenza virions were found at apical surfaces in duplicate samples examined at 8 h postinfection (25).

Surface expression of NA in influenza virus-infected cells. To investigate the site of surface expression of NA, it is necessary to expose both surfaces of a polarized monolayer to reagents for immunospecific labeling. Growing cells on micropore filters provided access to the membranes. We determined that cells grown on such filters maintained polarity, as evidenced by the preferential apical budding of influenza virions (Fig. 1B and C).

To determine the surface distribution of NA, MDCK cells grown on cellulose-nitrate filters were infected with either influenza virus or VSV, and the distribution of the viral glycoproteins on the basolateral and apical membranes was examined by immunoferritin labeling. Monoclonal antibodies to HA (Fig. 2B) as well as to NA (Fig. 2D) reacted with apical plasma membranes but not the basolateral membranes of influenza virus-infected MDCK cells. Under the same labeling conditions, antibodies to the VSV G protein were found to react with the basolateral surfaces of VSV-infected MDCK cells (Fig. 2C), and the basolateral membranes of MDBK cells infected with herpes simplex virus could be labeled by antibodies specific for herpesvirus glycoproteins (R. V. Srinivas, manuscript in preparation). Thus, the lack of ferritin labeling of the basolateral membranes in influenza virus-infected cells with anti-NA or anti-HA was not due to a lack of access to the basolateral membranes. Mock-infected MDCK cells labeled with an identical antibody regimen showed no labeling of apical or basal surfaces (Fig. 2A).

When AGMK or MA104 cells grown on micropore filters were infected with influenza virus and labeled with anti-NA and the colloidal gold-conjugated antibody, both virions and microvilli associated exclusively with the apical membranes were found to be labeled with gold particles (Fig. 3A and C). The basal membranes of these cells showed no labeling above background levels (Fig. 3B and D). Mock-infected AGMK cells labeled with an identical antibody regimen showed no labeling of apical (Fig. 3E) or basal (Fig. 3F) surfaces. Thus, NA, like HA, was found to be directionally transported to the apical membranes of polarized epithelial cells.

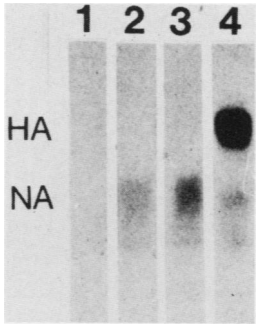

FIG. 4. SDS-polyacrylamide gel electrophoresis of whole virus and cloned NA in MA104 cells. MA104 cells were mock infected (lane 1) or infected with either SNC (lane 2), SN10 (lane 3), or influenza virus (lane 4). Cells were labeled with D-[2-^{3}H]mannose at the times indicated in the text. The cells were washed five times with cold PBS and lysed in cell dissociation buffer. The lysates were used for immunoprecipitation with formalinized *S. aureus* cells that had been incubated with rabbit anti-influenza A virus antiserum. The immunoprecipitates were electrophoresed through an SDS–10% polyacrylamide gel, and the protein bands were visualized by standard fluorographic techniques.

Expression of NA with SV40 vectors. To confirm that MA104 cells infected with the recombinant viruses were producing the influenza virus NA, cells were infected with SNC, SN10, or influenza virus. These cells were radiolabeled in vivo with [^{3}H]mannose and lysed, and the polypeptides were immunoprecipitated with *S. aureus* cells that had been incubated with antiserum to influenza virus. Both of the cloned genes produced polypeptides with an electrophoretic mobility similar to that of the NA of influenza virus (M_r, 56,000) (Fig. 4).

The distribution of NA on MA104 cells infected with each recombinant virus, as seen by immunofluorescence, is shown in Fig. 5. The SNC-infected cells displayed punctate, patchy fluorescence over their surfaces, similar to that observed with influenza virus-infected cells (Fig. 5A and C). MA104 cells infected with SN10 also displayed punctate fluorescence but in a more diffuse pattern (Fig. 5B). Quantitation of the observed surface-fluorescence-positive MA104 cells infected with SNC or SN10 virus stock indicated that ca. 20 and 30%, respectively, of the cells were infected and expressing the cloned glycoprotein gene. These percentages did not change markedly after further incuba-

FIG. 3. Polarity of expression of NA in cells infected with influenza virus. AGMK and MA104 cells were seeded separately onto preincubated micropore filters and infected with influenza virus or mock infected. At 5 to 6 h postinfection the infected cells and a sample of uninfected cells were reacted with primary and conjugated antibodies and then prepared for electron microscopy. (A and B) Apical (A) and basal (B) surfaces of influenza virus-infected AGMK cell labeled with anti-NA and colloidal gold conjugate (A, ×68,000); (B, ×40,000). (C and D) Apical (C) and basal (D) surfaces of influenza virus-infected MA104 cell labeled with anti-NA and colloidal gold conjugate (C, ×52,000); (D, ×60,000). (E and F) Apical (E) and basal (F) surfaces of mock-infected AGMK cells labeled with anti-NA and colloidal gold conjugate (×42,000).

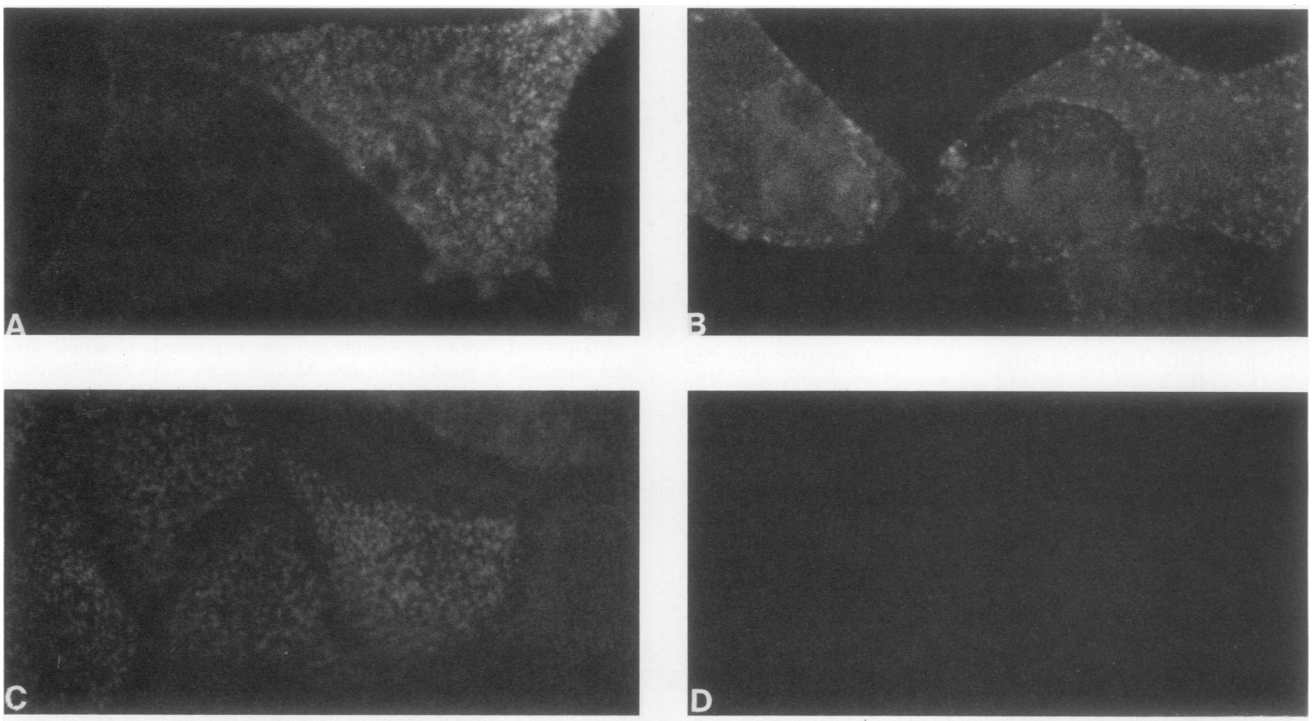

FIG. 5. Surface expression of whole virus and cloned NA in AGMK cells. Subconfluent monolayers of AGMK cells were infected with recombinant virus (36 h) or influenza virus (7 h). The cells were stained for surface fluorescence with anti-influenza virus rabbit immunoglobulins, followed by fluorescein-conjugated goat anti-rabbit IgG. Cells were infected with SNC (A), SN10 (B), or influenza virus (C) or mock infected (D).

tion (>48 h postinfection), in part due to increasing loss of cells in response to the lytic SV40 virus infection. Similar uorescence patterns were observed in infected AGMK cells (not shown).

Polarity of NA expressed from SV40 vectors. To examine whether NA is sorted to specific membranes in epithelial cells in the absence of other influenza virus proteins, AGMK and MA104 cells grown on micropore filters were infected with either SNC or SN10. At 48 h postinfection, these cells were incubated with monoclonal antibody to NA and then with the colloidal gold conjugate. Cells infected with SNC had small patches of gold particles along the apical membranes, most of which were associated with the microvilli (Fig. 6A and E). The basal membranes of these cells exhibited only background levels of gold particles, indicating that the SNC-encoded antigen was not present in these membranes (Fig. 6B and F). When cells infected with SN10 were immunolabeled and observed by electron microscopy, the differential distribution of gold particles on the apical and basolateral membranes was quite similar to that observed for SNC-infected cells (Fig. 6C and D). However, the particles were more randomly distributed on microvilli as well as on the more linear portions of the surfaces (Fig. 6). Quantitation of gold particles on either surface of the antigen-positive cells showed that 89% of the total adhering gold particles were bound to the apical membranes of SNC-infected cells (Table 1). When the gold particles on the surface of SN10-infected cells were counted, 95% were bound on the apical membranes (Table 1). These data indicate that the NA polypeptide contains structural features which result in its transport to the apical surfaces of polarized epithelial cells.

DISCUSSION

Precedent and perhaps requisite to the polarized budding of virions, viral envelope glycoproteins are differentially sorted to appropriate plasma membranes (20). One of the most extensively studied viral glycoproteins which exhibits this directed sorting is the HA of influenza A virus. The HA is expressed almost exclusively on the apical membranes of virus-infected cells (19, 25). Evidence has been obtained that the HA gene product, in the absence of other influenza viral proteins, is transported to and expressed on the apical membranes of polarized cells (25). Thus, the HA contains the information needed to direct its transport in the absence of other influenza virus proteins. This directed transport is not dependent on glycosylation (10, 11, 17, 26), indicating that the required information lies in the polypeptide portion of the glycoprotein. We have now investigated the site of surface expression of the NA gene of influenza virus. Since

FIG. 6. Immunogold labeling of recombinant virus-infected cells. AGMK and MA104 cells were grown on filters and infected with either SNC or SN10. At 48 h postinfection, the culture medium was removed, and the filters were rinsed repeatedly in PBS-BSA (pH 8.2). The filters were then incubated in monoclonal anti-NA (1:20) for 30 min at 20°C. After extensive washing with PBS-BSA, the filters were incubated in colloidal gold conjugate (1:5) for 30 min at 20°C. The filters were rinsed extensively in PBS, and the cells were fixed by 1% glutaraldehyde in PBS and embedded in epoxy resin for electron microscopy. (A and B) SNC-infected AGMK cell. (A) Apical surface (×54,000); (B) basal surface (×56,000); (C and D) SN10-infected AGMK cell. (C) Apical surface (×80,000); (D) basal surface (×60,000). (E and F) SNC-infected MA104 cell. (E) Apical surface (×56,000); (F) basal surface (×54,000).

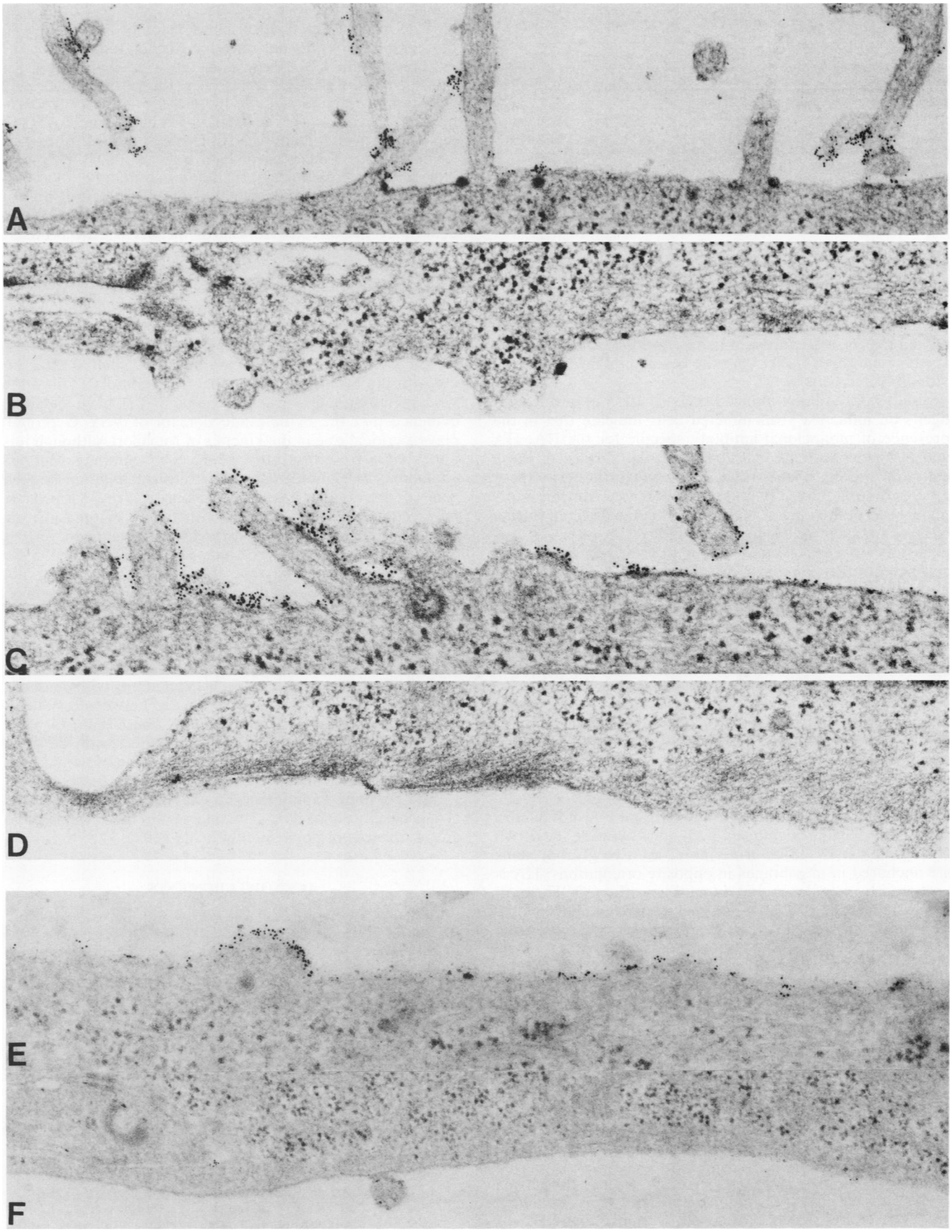
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TABLE 1. Preferential immunogold labeling of apical membranes of recombinant virus-infected cells[a]

Virus vector	Colloidal gold grains[b]			
	Total no.	No. on apical membranes	Avg no./cell	% on apical membrane
SNC	3,843	3,424	137	89
SN10	4,240	4,035	151	95

[a] AGMK cells were grown, infected, and prepared for electron microscopy as described in the legend to Fig. 6.

[b] The number of colloidal gold grains on each side of the cells was counted and recorded in conjunction with the number of cells counted.

influenza virions bud from the apical membranes of polarized cells, it was expected that NA would also be present on these surfaces; however, it was not known whether the NA was also distributed on the basolateral surfaces. We observed that NA is expressed almost exclusively on the free apical membranes rather than the basolateral membranes of virus-infected cells.

Studies of antibody-induced redistribution of surface antigens on influenza virus-infected cells indicate that in the presence of monoclonal antibody specific for the HA, HA and NA glycoproteins will co-cap on the surface of these cells (S. Basak, unpublished data), suggesting that these glycoproteins may be closely associated during morphogenesis of the virus. To establish that the directed sorting of NA can occur independently of the sorting of HA, it was necessary to express the NA in a polarized cell in the absence of other influenza virus proteins. This was accomplished by the use of NA cDNA cloned into SV40. We observed that when polarized monkey kidney cells grown on filters were infected with SV40 recombinant viruses carrying the complete gene or a truncated gene of influenza virus NA, the cloned gene product was expressed preferentially on free apical membranes. These results indicate that the NA polypeptide, independent of the HA polypeptide, contains structural features sufficient for directed transport in polarized cells. Furthermore, since it has already been established that glycosylation does not play a role in the polarity of virus maturation, we suggest that this sorting information resides in the polypeptide backbone of this glycoprotein.

The amino acid sequences of the HA and NA of influenza A viruses reveal no significant homology between these two glycoproteins (13, 18). Furthermore, the two glycoproteins are anchored in membranes in opposite orientations: HA is anchored by a hydrophobic domain near the COOH terminus, whereas NA is anchored by an uncleaved hydrophobic sequence near the NH_2 terminus (5, 9, 13). Therefore, the finding that these two different proteins are directed to the same surface of polarized cells indicates that the cellular machinery is capable of recognizing more than one type of signal for transport to apical cell surfaces.

Efforts to locate the regions of the glycoproteins that contain the information for sorting center around the membrane-spanning region and the cytoplasmic domain. Chimeric glycoprotein genes containing the majority of the influenza virus HA sequences with either the NH_2- or the COOH-terminal sequences of the G protein of VSV yielded glycoproteins that were successfully translocated into the rough endoplasmic reticulum and primarily glycosylated; however, these chimeric glycoproteins were not transported to the Golgi complex and therefore never reached the plasma membranes (16). By using chimeric glycoproteins containing the NH_2 terminus of NA followed by signal-minus HA or signal-minus, anchor-minus HA, Bos and co-workers (6) have shown that the NH_2-terminal region of NA (residues 1 through 40) provides not only the anchor function for the mature glycoprotein but also the signal function for translocation. Recently, it has been shown that the first 12 NH_2-terminal amino acids of the N1 and N2 NA glycoproteins are strictly conserved in all type A influenza viruses (4); the cytoplasmic domain of NA possibly consists of the first 6 amino acids. The present data indicate that the first 10 NH_2-terminal amino acids, including the putative cytoplasmic domain, may not be needed in directing the transport of the polypeptide to a specific membrane domain, as evidenced by the directed sorting of the SN10 gene product. SN10 differs from SNC in that the first 10 NH_2-terminal amino acids are replaced by 9 amino acids encoded by the SV40 agnogene and linker sequences (8). Either the replaced NH_2-terminal sequence in SN10 provides a function similar to that of SNC, or this domain does not play a critical role in sorting. It is possible that the conserved residues at the NH_2 terminus of NA may play some other role, such as interacting with the M protein during incorporation of NA into the viral membrane. Rose and Bergmann (22) have obtained evidence that the cytoplasmic domain of VSV G protein plays a critical role in the kinetics of transport, although it is not essential for transport to the plasma membrane. Mutants containing a deletion of the H-2L^d transplantation antigen genes of mice (either missing the majority of the cytoplasmic tail or containing short replacements for the cytoplasmic tail sequence) transported and expressed the antigen on the plasma membrane with the same kinetics as the wild-type antigen (28), indicating that the cytoplasmic tail is not involved in the transport of this protein. We have, however, noted an apparent difference in the pattern of immunofluorescence and immunogold labeling between SNC and SN10 polypeptides, suggesting an inherent difference in the mobility of these gene products.

Markoff and co-workers (15), using mutants with deletions in the NA gene, have found that a region between residues 7 and 28 is necessary for translocation. These data, together with the present results, suggest that the region between residues 11 and 28 is critical for translocation of the nascent glycoprotein and for complete glycosylation. Consequently, we are pursuing experiments to examine this region of the NA gene more closely in relation to directed transport to apical surfaces of polarized epithelial cells.

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