Characterization of the Influenza Virus M_2 Integral Membrane Protein and Expression at the Infected-Cell Surface from Cloned cDNA

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An investigation of properties of the influenza A virus M_2 protein indicated that it is synthesized by 2 h postinfection together with other viral polypeptides and is transported to the infected-cell surface with a half-time of approximately 30 to 40 min. The available evidence suggests that M_2 is not N-glycosylated even though it contains a potential glycosylation site, and the intracellular pattern of protein distribution includes localization to the Golgi apparatus. Proteolysis of intracellular microsome vesicles followed by immunoprecipitation with antiserum to a synthetic oligopeptide indicated that the M_2 protein contains an extensive region of COOH-terminal amino acids exposed on the cytoplasmic side of the infected-cell membrane. A cDNA clone of the M_2 mRNA was obtained and expressed in an SV40 recombinant vector. The M_2 protein expressed by the vector became associated with the Golgi complex and was found on the surface of vector-infected cells. M_2 is antigenically conserved among all strains of influenza virus both in regions exposed on the cell surface and intracellularly.

The eight single-stranded genomic RNA segments of influenza A virus code for 10 or more polypeptides (for reviews, see references 11 and 15). RNA segment 7 encodes two known polypeptides: the viral membrane or matrix protein (M_1) and a protein of apparent $M_r \cong 15,000$ designated M_2 (14). The mRNA-encoding polypeptide M_1 is colinear with RNA segment 7, whereas M_2 is encoded by a spliced mRNA (20). The nucleotide sequence of the M_2 mRNA predicts that M_1 and M_2 share the initiator methionine residue and eight following NH_2-terminal residues. The remaining 88 amino acids of M_2, encoded after the 3' splice junction on the mRNA, are derived from the second open reading frame of RNA segment 7 (20).

Examination of the predicted protein sequence of M_2 suggested that it contains a region of amino acids (residues 25 to 43) that is sufficiently hydrophobic to interact with membranes (20, 21). We have recently presented evidence that M_2, in addition to hemagglutinin (HA) and neuraminidase (NA), is an integral membrane protein that is abundantly expressed at the infected cell surface (21). However, unlike HA and NA, our available evidence indicates that the M_2 polypeptide does not become incorporated into virions (14; S. L. Zebedee and R. A. Lamb, unpublished observations). To demonstrate the orientation of M_2 in membranes, we prepared two site-specific oligopeptide antisera: one to the NH_2-terminal residues 2 to 10 (SP1 antiserum) and the other to the COOH-terminal-region residues 69 to 79 (SP2 antiserum) (21). Trypsin treatment of infected cells and immunoprecipitation with the SP2 antiserum indicate that a minimum of 18 NH_2-terminal amino acids of M_2 are exposed at the cell surface. In addition, the SP1 antibody can recognize the M_2 protein on the infected-cell surface (21). Interestingly, M_2 residues 1 to 10 are conserved in the predicted sequence of M_2 in the human and avian influenza virus strains for which the nucleotide sequence of RNA segment 7 is known (A/PR/8/34 [1, 39], A/Udorn/72 [17], A/Bangkok/1/79 [28], A/FPV/Rostock/34 [24]).

In this paper, we describe experiments to further investigate the properties of M_2, its association with subcellular membranes, and expression of a cDNA copy of the M_2 mRNA in a eucaryotic vector.

MATERIALS AND METHODS

Viruses and cells. Influenza viruses (A/WSN/33 and A/Udorn/72) were grown as previously described (13). Avian strains of influenza A virus, kindly provided by V. S. Hinshaw and R. G. Webster, were grown in embryonated eggs. CV1 and MDCK cells were passaged and infected as described previously (12, 16).

Isotopic labeling of infected-cell lysates, immunoprecipitation, and polyacrylamide gel electrophoresis. Influenza virus-infected cells were labeled except where stated with 50 to 100 μCi of [^{35}S]cysteine at 4.5 to 5 h postinfection (p.i.) for 2 h in cysteine-deficient Dulbecco modified Eagle medium or with 200 μCi of [^{3}H]glucosamine at 4.5 h p.i. for 2 h in phosphate-buffered saline. Cells infected with simian virus 40 (SV40) recombinant viruses were labeled for 4 h with 100 μCi of [^{35}S]cysteine at 40 to 48 h p.i. Antibodies specific for the M_2 protein (SP1, SP2, and DM2) were prepared as described previously (21) and were used for immunoprecipitation of M_2 as described previously (16, 21). Samples were analyzed by electrophoresis on 17.5% polyacrylamide gels containing 4 M urea (21).

Microsome isolation and proteolysis. To prepare microsome fractions, [^{35}S]cysteine-labeled infected CV1 cells were washed twice with phosphate-buffered saline, harvested, and pelleted at 1,000 × g for 5 min. Cells were suspended in 6 ml of hypertonic buffer (0.001 M $MgCl_2$, 0.01 M Tris hydrochloride [pH 7.5]) and allowed to swell on ice for 5 min before Dounce homogenization. The disrupted cells were divided into 2-ml aliquots and incubated at 37°C
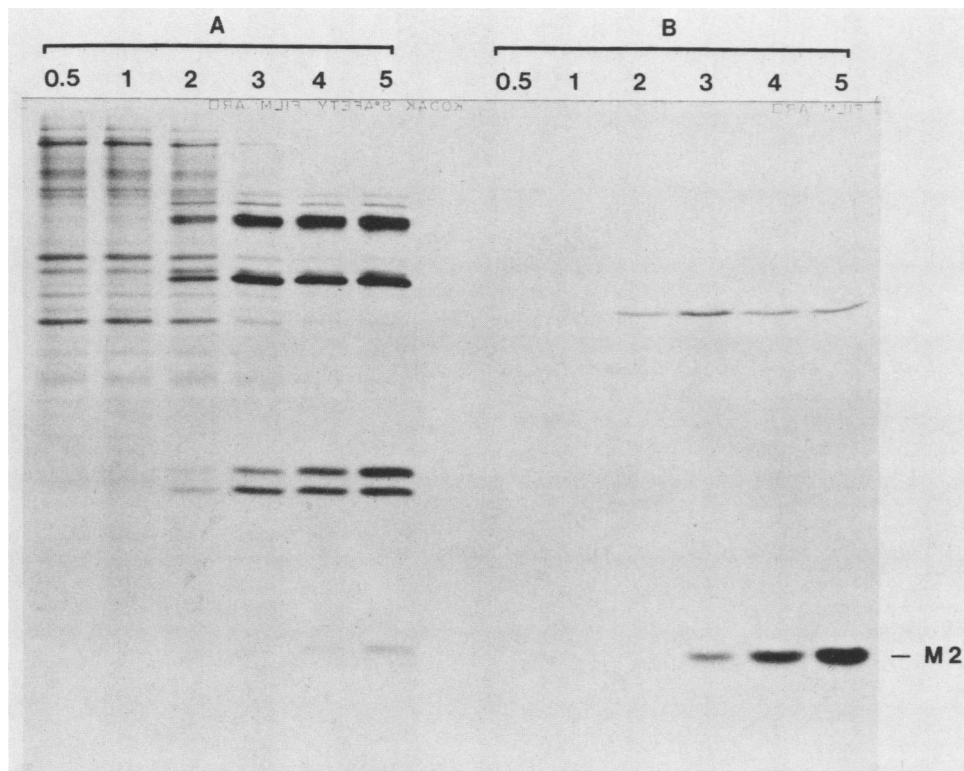
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FIG. 1. Time course of appearance of M_2 in infected cells. CV1 cells were infected with influenza A/WSN/33 virus, and at the hours p.i. indicated were labeled with [^{35}S]cysteine for 30 min and lysed in RIPA buffer as described previously (16). (A) Direct lysates; (B) immunoprecipitations with antiserum to the SP2 peptide. Under the conditions used a small amount of NP precipitated nonspecifically as observed previously (16, 21).

for 1 h in the presence of tosylamide-phenylmethyl chloromethyl ketone (TPCK)-treated trypsin (200 μg/ml), TPCK-trypsin plus 1% Nonidet P-40, or without protease. Trypsin was inhibited by the addition of aprotinin, and microsomes were pelleted through a 10% sucrose gradient as described previously (8). Trypsin treatment of the surface of [^{35}S]cysteine-labeled infected CV1 cells was done as described previously (21).

Indirect immunofluorescence. For surface labeling, cells were fixed in 2% formaldehyde. In some experiments, after formaldehyde fixation cells were permeabilized by treatment with acetone at −20°C for 5 min. Indirect immunofluorescence was done as described previously (4). The antibody to polyacrylamide gel-isolated M_2 (DM2), which slightly cross-reacts with a CV1 cell protein (21), was purified before use in immunofluorescence. The immunoglobulin G (IgG) fraction was prepared by protein A-Sepharose chromatography (31), and the eluted IgG was adsorbed by five sequential incubations with uninfected CV1 cells which had been grown, fixed, and permeabilized in glass flasks. The adsorbed antiserum was found to be specific for M_2 and did not react with uninfected CV1 cells. Monoclonal antibodies to WSN HA (ascites fluid) were kindly provided by K. van Wyke. Wheat germ agglutinin (WGA) was coupled to rhodamine and used as a stain for the Golgi apparatus (35).

Preparation of the M_2 cDNA clone. Cytoplasmic poly(A)-containing mRNAs were isolated from influenza virus A/Udorn/72-infected HeLa cells and separated on sucrose gradients as described previously (13, 16). Fractions from the top of the gradient were translated in wheat germ extracts, and those yielding the M_2 polypeptide (20) were

pooled and used as an enriched mRNA fraction for the synthesis of cDNAs to the M_2 mRNA. First-strand synthesis was done with oligo(dT) as a primer for reverse transcriptase, using conditions described previously (29). To increase the probability of obtaining full-length double-stranded cDNA copies, the second strand synthesis was primed with the synthetic dodecanucleotide d(5'-AGCAAAAGCAGG-3'), which is complementary to the 3' end of all influenza virus RNA segments, and the primer was extended with reverse transcriptase. Double-stranded cDNAs were treated with nuclease S1, and BamHI linkers were added. The double-stranded cDNAs were ligated to BamHI-digested and bacterial alkaline phosphatase-treated pBR322. Escherichia coli DH1 cells were then transformed with the recombinant plasmids, and ampicillin-resistant colonies were screened by hybridization with a ^{32}P-labeled cDNA to influenza virus RNA segment 7 (pM/45) (17). The plasmid DNA in positive colonies was digested with various restriction endonucleases, and the DNA fragment sizes were compared with those predicted from the known nucleotide sequence (20). Finally, the M_2 cDNAs were confirmed by direct nucleotide sequencing with base-specific chemical cleavages (23).

Construction of SV40-M_2 recombinant virus. The SV40 VP1 replacement vector used in these experiments was analogous to one described previously (25). It was chosen because of the small size of the M_2 cDNA and the minimal packaging requirements for the SV40 virion in a lytic infection. The SV40 late region encoding the initiation codon and most of VP1 was removed from SV40 DNA by partial HindIII digestion to linearize the molecule at nucleotide
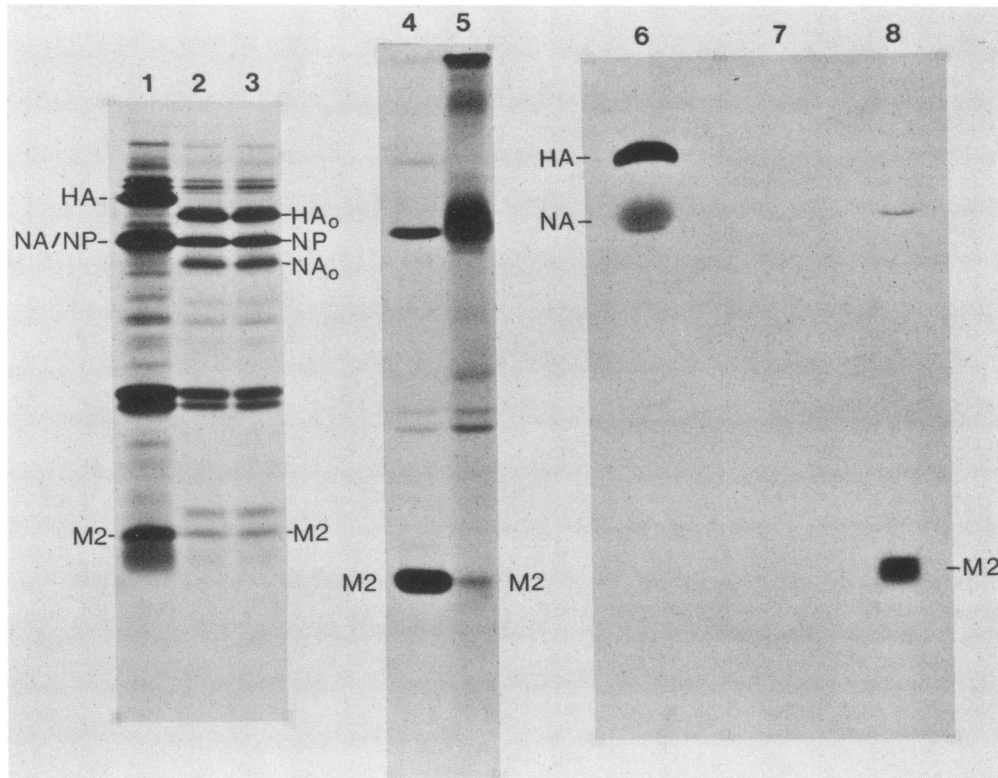
FIG. 2. Evidence for lack of glycosylation of M_2 protein in infected cells. Lanes 1 to 3: WSN-infected CV1 cells were treated at 3 h p.i. with tunicamycin and labeled with [^{35}S]cysteine at 5 h p.i., and lysates examined on polyacrylamide gels. Lane 1, no treatment; lane 2, 0.5 µg of tunicamycin per ml; lane 3, 1.0 µg of tunicamycin per ml. Lanes 4 and 5: Comparison of mobility of M_2 synthesized in vivo and in vitro. Lane 4, immunoprecipitation of [^{35}S]cysteine-labeled WSN-infected CV1 cells with SP2 antiserum; lane 5, immunoprecipitation with SP2 antiserum of [^{35}S]cysteine-labeled polypeptides synthesized in wheat germ extracts programmed with poly A-containing mRNAs from WSN-infected HeLa cells. Lanes 6 to 8: Labeling of WSN-infected CV1 cells with [^{3}H]glucosamine. Lane 6, immunoprecipitation with antiserum to purified WSN virions; lane 7, immunoprecipitation with anti-M_2 SP2 serum with 20 times the amount of antigen as in lane 6; lane 8, immunoprecipitation with the SP2 antiserum of M_2 from [^{35}S]cysteine-labeled WSN-infected cells. The diffuseness of the M_2 band observed on the autoradiograph of the gel shown in lanes 1 to 3 is a property of M_2 previously observed (21). NP, Nucleocapsid protein. HA_0 and NA_0, Unglycosylated HA and NA, respectively.

1493, followed by the addition of BamHI linkers and then BamHI digestion. The large DNA fragment containing all of SV40 except nucleotides 1493 to 2533 (SV numbering system, see reference 3) was used as a vector into which the M_2 cDNA was inserted, such that the M_2 cDNA was under the control of the SV40 late-region promoter, splicing, and polyadenylation signals. To facilitate the bacterial cloning, the unique TaqI site of SV40 was converted into an XbaI site and cloned into a modified pBR322 plasmid containing an XbaI site as described previously (18, 19). The SV40-M_2 DNA was introduced into CV1 cells by DEAE-dextran-mediated transfection together with DNA of an SV40 early region deletion mutant (dl 1055) (30) to act as a helper virus. Lytic stocks were prepared as described previously (18). The expression of M_2 was examined by [^{35}S]cysteine-labeling and immunoprecipitation. SV40-M_2 recombinant virus stocks were analyzed for the lack of DNA rearrangements by extraction of small DNAs from infected cells (9), restriction enzyme digestions, and DNA blots.

RESULTS

Synthesis of M_2 in influenza virus-infected cells. Although it is known that influenza virus M_2 polypeptide is synthesized from a small spliced mRNA and that M_2 becomes inserted into the host-cell plasma membrane (14, 21), the time course

of appearance of M_2 in infected cells after infection has not been examined. CV1 cells were infected with strain A/WSN/33 and labeled with [^{35}S]cysteine at various times after infection. Small amounts of M_2 could be detected between 2 and 3 h p.i. from cell lysates examined either directly or after immunoprecipitation, concurrent with the shutdown of host-cell protein synthesis (Fig. 1). The rate of synthesis of M_2 was similar to that of M_1 and did not have a greatly different kinetic pattern from the other viral polypeptides. To investigate the stability of M_2 in infected cells, we performed a pulse-chase analysis. At 5 h p.i. cells were labeled with [^{35}S]cysteine for 15 min, followed by a 3-h chase period in unlabeled medium. The data indicate that M_2 is a stable protein (data not shown).

Evidence for the lack of glycosylation of M_2. The predicted amino acid sequence of M_2 (20, 21) indicates the presence of a potential site (Asn-Asp-Ser, residues 20 to 22) for N-linked glycosylation located near the known minimal region of M_2 that is exposed at the cell surface. To examine for the presence of oligosaccharides on the M_2 protein, we used three methods of analysis: (i) treatment of infected cells with tunicamycin, (ii) translation of influenza virus mRNAs in vitro, and (iii) labeling of infected cells with [^{3}H]glucosamine.

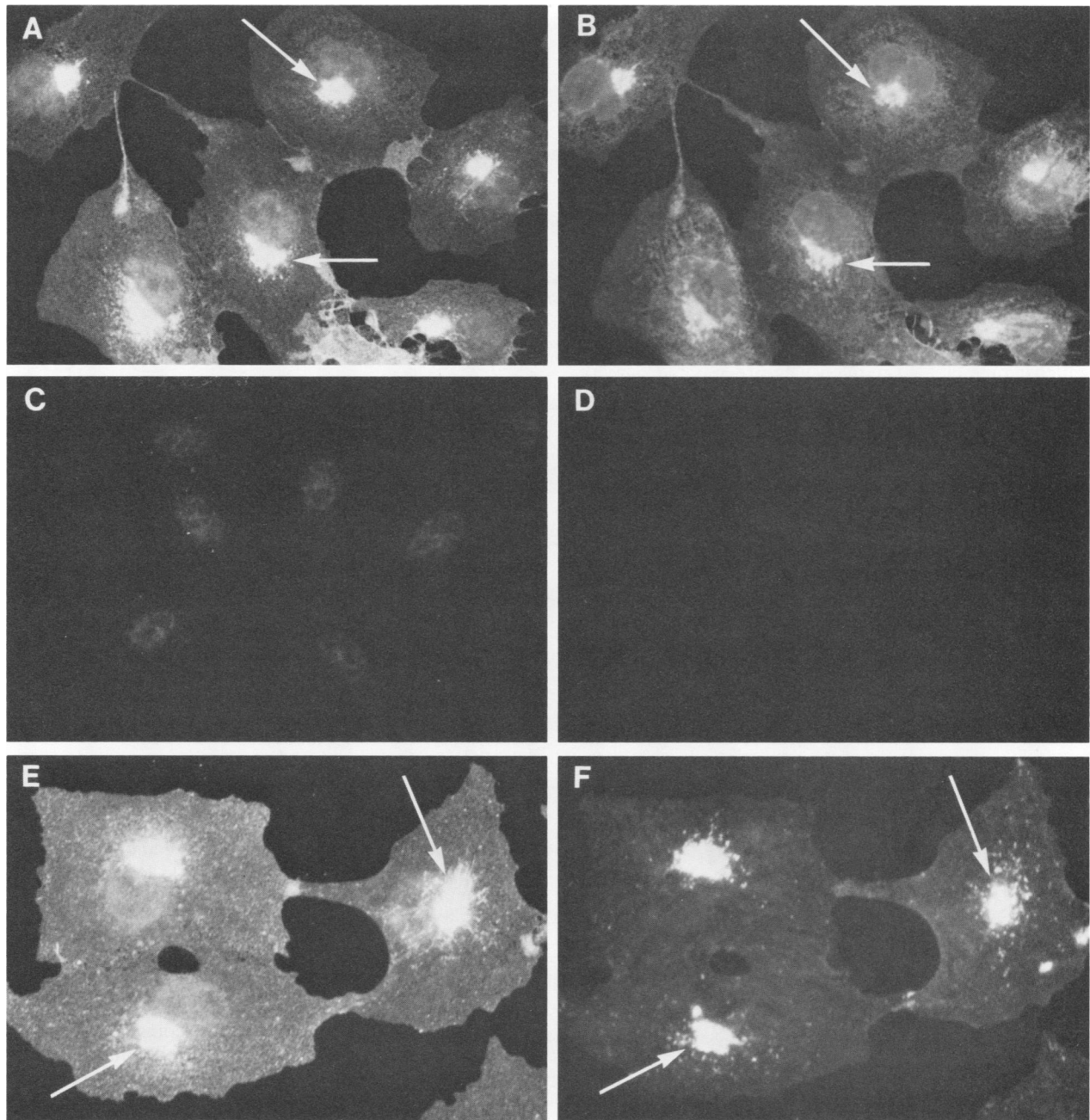Infected cells were treated with 0.5 µg of tunicamycin per

FIG. 3. Indirect immunofluorescence detects M₂ in the Golgi complex. CV1 cells were mock infected or infected with the WSN strain of influenza virus, and at 5 h p.i. they were fixed in 2% formaldehyde for 30 min and permeabilized in acetone at −20°C for 5 min. The upper panels show the same field of virus-infected cells stained sequentially with purified rabbit IgG to gel-denatured M₂ protein (DM2, purified as described in Materials and Methods) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (panel A), or after staining with a mixture of monoclonal antibodies (ascites fluid) to influenza virus HA and tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG (panel B). Panels C and D are mock-infected cells stained in the same manner as A and B, respectively. The bottom panels show the pattern of M₂ staining in WSN-infected cells, using antiserum to the gel-denatured M₂ (as in panel A) and FITC-conjugated goat anti-rabbit IgG (panel E) tetramethylrhodamine isothiocyanate-conjugated WGA (panel F). Arrows indicate the Golgi complex. Exposure times for panels C and D were manually adjusted to be the same as for panels A and B, respectively.

ml, an inhibitor of N-linked glycosylation (34), before labeling with [³⁵S]cysteine. The mobility of M₂ was unchanged (Fig. 2, lane 2) compared with that of untreated control infected cells (Fig. 2, lane 1). The effectiveness of the tunicamycin treatment can be seen from the changes in mobility in HA and NA between the untreated (lane 1) and

treated (lane 2) cells. We would have expected, as M₂ is a small polypeptide (apparent $M_r \cong 15,000$), that the addition of one oligosaccharide chain would cause a significant alteration in the mobility of the polypeptide.

An indirect means of examining for the addition of carbohydrates on M₂ in either an N-linkage or O-linkage is to

compare the electrophoretic mobility of M_2 synthesized in vivo and in vitro, since glycosylation of newly synthesized polypeptides does not occur in wheat germ extracts. mRNAs were isolated from influenza virus-infected HeLa cells, translated in vitro, and immunoprecipited with the M_2 SP2 antiserum. M_2 synthesized in vitro (Fig. 2, lane 5) had the same mobility as M_2 immunoprecipitated from infected cells (Fig. 2, lane 4). The major nonspecific band seen in the immunoprecipitate of the in vitro products is free [^{35}S]cysteine exogenously labeling the immunoglobulin heavy chain.

Further evidence for the lack of oligosaccharide addition was obtained by labeling WSN-infected cells with [^{3}H]glucosamine. Figure 2, lane 6, shows immunoprecipitation of [^{3}H]glucosamine-labeled HA and NA with anti-WSN serum. In Fig. 2, lane 7, 20 times the amount of [^{3}H]glucosamine-labeled antigen was precipitated with the SP2 M_2 antiserum, yet no band in the position of M_2 was detected. Figure 2, lane 8, shows a [^{35}S]cysteine-labeled M_2 immunoprecipitate as a marker lane.

M_2 is transported to the cell surface through the Golgi complex and in microsomal vesicles. Our previous data indicate that M_2 is anchored in the plasma membrane by an internal hydrophobic domain (21), and it is therefore of interest to examine the association of M_2 with intracellular organelles. The internal cellular location of M_2 was compared with that of HA, a well-characterized integral membrane protein, by indirect immunofluorescence on fixed, permeabilzed cells. Figure 3 shows examples of the intracellular immunofluorescence staining pattern of fluorescein-
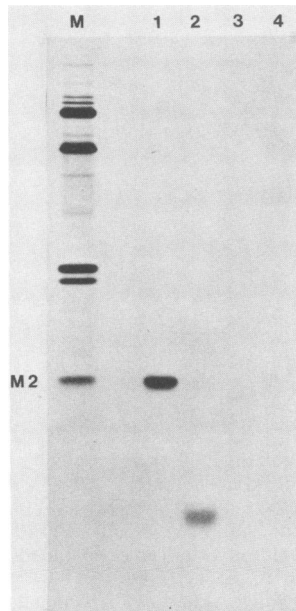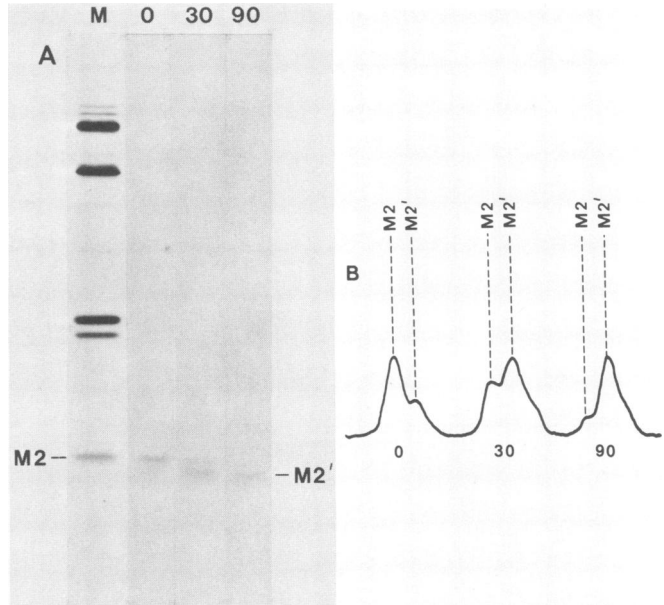

FIG. 5. Rate of appearance of M_2 on the infected-cell surface. (A) At 5 h p.i., WSN-infected CV1 cells were labeled for 15 min with [^{35}S]cysteine and then incubated with unlabeled medium (chase). At the indicated times (minutes), the cells were harvested and subjected to trypsin digestion. Samples were immunoprecipitated with antiserum against the COOH region of M_2 (SP2). M, Direct lysate of WSN-infected cells as a marker. (B) Densitometer scanning of the corresponding autoradiogram shown in panel A. M_2', Trimmed form of M_2.

FIG. 4. Proteolysis of the COOH terminal region of M_2 in infected-cell microsomes. Microsome preparations from [^{35}S]cysteine-labeled influenza virus-infected CV1 cells were not treated (lane 1) or were treated with trypsin in the absence (lanes 2 and 3) or presence (lane 4) of Nonidet P-40 as described in Materials and Methods. Samples were immunoprecipitated with antiserum to SP1 which recognizes the NH_2 terminus (lanes 1, 2, and 4) or antiserum to the COOH-terminal region (SP2) of M_2 (lane 3). M, Marker polypeptides from WSN-infected CV1 cells after labeling with [^{35}S]cysteine.

labeled M_2 (panel A) and rhodamine-labeled HA (panel B) in the same field of WSN-infected cells. Antibodies to both proteins were found to stain similar regions of infected cells, which include cytoplasmic fluorescence and intense staining of a perinuclear structure. Panels C and D show the control experiment of treating uninfected cells with the same antibodies as in panels A and B, respectively. The immunofluroescence staining in the same field of infected cells with fluorescein-labeled M_2 antibody (panel E) or rhodamine-labeled WGA (panel F), a lectin which has been shown previously to preferentially bind to the Golgi complex (35), demonstrate fluorescence in a coincident perinuclear structure. Thus, these data suggest that intracellular M_2 associates with the Golgi apparatus.

The presence of M_2 in the Golgi apparatus suggests that M_2 is transported to the cell surface by a vesicular transport mechanism. Although our previous experiments indicated that 18 to 23 N-terminal residues are exposed at the cell surface, the presence of a cytoplasmic tail of up to 54 COOH-terminal amino acids has not been demonstrated. A crude preparation of microsomes from [^{35}S]cysteine-labeled infected cells was isolated, treated with trypsin, and pelleted through a sucrose gradient. The site-specific M_2 antiserum for either the N terminus (SP1) or the C terminus (SP2) was used in immunoprecipitation assays of the protease-treated microsomes. The SP1 antiserum precipitated a band of $M_r \cong$ 7,000 (Fig. 4, lane 2), but this band was not found after immunoprecipitation with SP2 antiserum (lane 3) or after trypsin treatment in the presence of 1% Nonidet P-40 to permeabilize the microsomes and precipitation with the SP1 antiserum (lane 4). A small amount of M_2 of the size expected when the protein is trimmed by protease at the cell
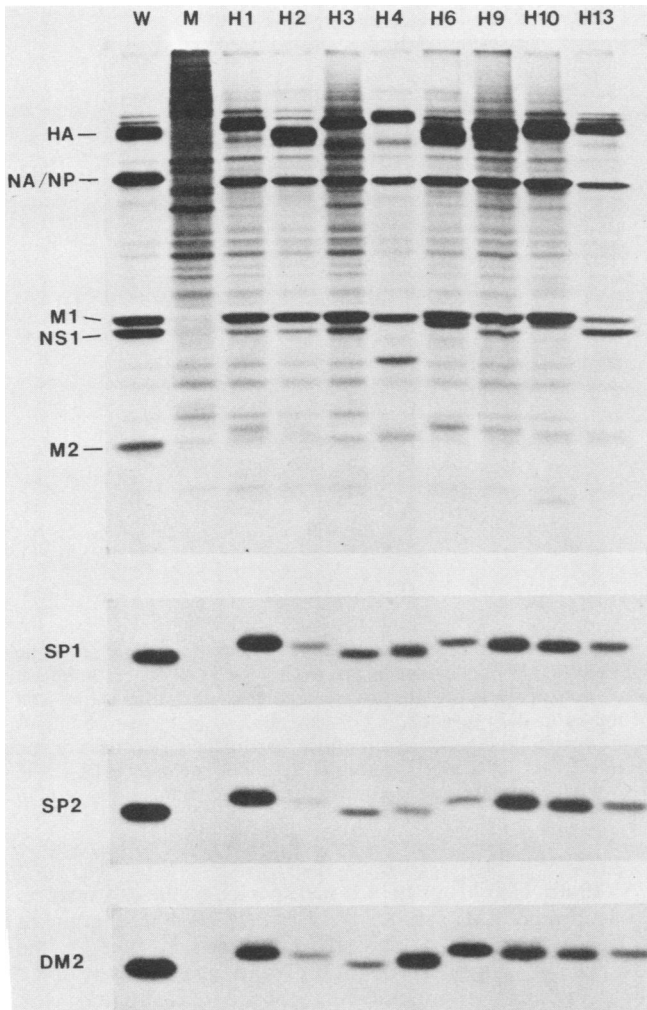
FIG. 6. Immunoprecipitation of M₂ from cells infected with various strains of avian influenza A virus. MDCK cells were infected with various subtypes of avian influenza viruses and at 4 h p.i. were labeled with [³⁵S]cysteine. The top panel shows direct lysates of the influenza virus-infected cells. The virus-specific polypeptides are indicated for the migration of polypeptides of the A/WSN/33 strain (lane W). Immunoprecipitation with M₂ antisera (21) was done as described previously (21) for synthetic oligopeptide antisera SP1 and SP2, and for antiserum to polyacrylamide gel-purified M₂ protein (DM2), as decribed previously (5). For the immunoprecipitations, the autoradiograph of the gel showing the M₂ region only is displayed. M, Mock-infected cells. Strains of avian influenza A virus are: H1, A/duck/Alberta/35/76(H1N1); H2, A/duck/Germany/1215/73(H2N3); H3, A/duck/Ukraine/1/63(H3N8); H4, A/duck/Czechoslovakia/56(H4N6); H6, A/turkey/Massachusetts/3740/65(H6N2); H9, A/turkey/Wisconsin/1/66(H9N2); H10, A/chick/Germany/N/49(H10N7); H13, A/gull/Maryland/704/77 (H13N6).

surface (M₂') can be observed in lane 3, indicating that a very small proportion of the microsomal preparation was contaminated by released plasma membrane vesicles. The use of M₂ site-specific antisera in immunoprecipitation experiments of protease-treated microsomes indicated that the N terminus and hydrophobic domain of M₂ are protected from proteolysis and that a large portion of the C-terminal region of the molecule, which includes the region recognized by the

SP2 antiserum, is exposed and accessible to trypsin cleavage.

To examine the rate of transport of M₂ to the infected-cell surface, we used a short pulse-label of [³⁵S]cysteine at 5 h p.i. followed by a chase period in unlabeled medium. At various times, the cells were harvested, incubated with trypsin to trim M₂ at the cell surface, washed to remove residual trypsin, and solubilized. Samples were immunoprecipitated with the C-terminal region antiserum (SP2) and analyzed on gels. The autoradiograph and densitometer tracings of the autoradiograph are shown in Fig. 5. Immediately after the labeling period (15 min), most of the newly synthesized M₂ was not trimmed by protease. Within 30 min, approximately 50% of the labeled M₂ was present on the cell surface and sensitive to proteolysis as shown by an approximately equal distribution of radioactivity in M₂ and trimmed M₂ (M₂'). At 90 min after the labeling period, a majority of M₂ was cleaved by trypsin to the smaller trimmed M₂' polypeptide. These data indicate that M₂ is transported to the cell surface with a half-time of approximately 30 to 40 min.

Conservation of antigenic sites in M₂ among influenza A virus subtypes. As discussed above, the predicted NH₂-terminal 10 amino acid residues of M₂ are conserved in the strains of influenza virus for which the nucleotide sequence of RNA segment 7 is known: A/PR/8/34, A/FPV/Rostock/34, A/Udorn/72, and A/Bangkok/1/79 (1, 17, 24, 28, 39). We have shown that antiserum to the NH₂ terminus (SP1) of the A/Udorn/72 strain precipitates M₂ from cells infected with the human virus isolates A/PR/8/34, A/WSN/33,
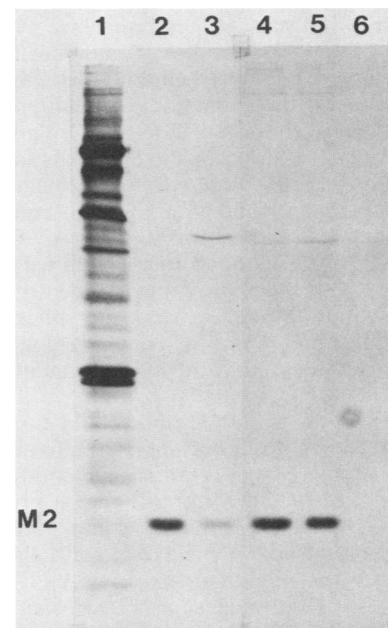

FIG. 7. Expression of the M₂ protein from the SV40-M₂ vector. [³⁵S]cysteine-labeled infected CV1 cells were immunoprecipitated with SP1 antiserum (lanes 2, 4, and 6) or SP2 antiserum (lanes 3 and 5). Lanes: 2 and 3, cells infected with A/Udorn/72 influenza virus; 4 and 5, cells infected with SV40-M₂ recombinant virus with M₂ DNA in the mRNA sense; 6, infection with M₂ DNA in the vRNA sense with respect to late SV40 transcription. Lane 1 is a direct lysate of A/Udorn/72-infected CV1 cells. A small amount of nonspecific precipitation of NP in lanes 2 and 3 and VP1 from the SV40 helper virus was observed as has been described previously (19, 21).

A/Udorn/72, A/Hong Kong/75, A/FW/1/50, and A/USSR/90/77 (21; our unpublished observations). We investigated further whether the NH$_2$-terminal region of M$_2$ can be recognized by the SP1 antiserum for many avian subtypes of influenza virus. MDCK cells were infected with a variety of avian influenza viruses from H1 to H13 subtypes and labeled with [^{35}S]cysteine. Figure 6 shows the patterns of polypeptides synthesized in cells infected with the various strains. The strain-specific migration of M$_2$ described previously for human influenza virus subtypes (14) can be observed. The SP1 antiserum was capable of recognizing M$_2$ from all the subtypes tested (Fig. 6), suggesting that the N-terminal region is antigenically conserved. We also tested whether the antiserum to the C-terminal region (SP2) and the antiserum to the denatured M$_2$ protein (DM2) could recognize the M$_2$ protein of the avian subtypes. Both the sequence-specific SP2 antiserum and the DM2 antiserum could recognize M$_2$ from all the strains tested (Fig. 6), although different amounts of M$_2$ were precipitated between antisera for a given strain (e.g., H6 strain), suggesting some degree of amino acid changes in the protein sequence. The only strain tested for which antiserum to the SP2 synthetic peptide, which was synthesized to the sequence of the A/Udorn/72 strain, did not precipitate M$_2$ was A/PR/8/34 (21). From the nucleotide sequences, it is known that there is a substitution of Lys for Glu at residue 70 (17, 39), and this change may alter the recognition of the SP2 antipeptide serum for the M$_2$ protein. Thus, although the complete sequence of M$_2$ is known for only four strains of influenza virus, the data described here suggest that the protein has regions that are antigenically conserved.

Expression of a cDNA clone encoding M$_2$ protein in eucaryotic cells. To further examine the synthesis and transport of the M$_2$ protein to the plasma membrane, we synthesized a cDNA to the M$_2$ mRNA. Poly(A)-containing RNAs from A/Udorn/72-infected cells were enriched for small mRNAs on sucrose gradients, and those fractions that yielded M$_2$ on translation in vitro were pooled. cDNAs were prepared with reverse transcriptase as described in Materials and Methods. Candidate cDNAs for M$_2$ were verified through a comparison of the splice junction and 5' and 3' end sequences (data not shown) to those predicted for M$_2$ mRNA (20). A cDNA clone (M$_2$ DNA) was obtained that contained the M$_2$ sequences with RNA segment 7 nucleotide 51 covalently linked to nucleotide 740 (the 5' and 3' splice sites) and containing the entirety of the M$_2$-coding region. The clone lacked only nucleotides 1 to 5 of RNA segment 7 at the 5' end of the mRNA.

To determine if the M$_2$ cDNA clone could express the M$_2$ protein in eucaryotic cells, it was inserted into an SV40 VP1 replacement vector (i.e., between SV nucleotides 1493 and 2533) under control of the SV40 late-region promoter and polyadenylation signal (see Materials and Methods). The recombinant SV40-M$_2$ DNA molecules with the M$_2$ DNA inserted in either the mRNA or viral RNA (vRNA) sense with respect to SV40 late-region transcription were transfected into CV1 cells, together with an SV40 early-region deletion mutant (dl 1055) (30), and lytic stocks of virus were prepared. When the SV40-M$_2$ (mRNA sense)-infected cells were labeled with [^{35}S]cysteine and immunoprecipitated with the SP1 or SP2 antiserum (Fig. 7, lanes 4 and 5), a band with mobility identical to that of M$_2$ precipitated from influenza virus A/Udorn/72-infected cells (Fig. 7, lanes 2 and 3) was detected. Recombinant virus stocks containing M$_2$ in the vRNA sense with respect to SV40 late-region transcription did not synthesize any M$_2$, as expected (Fig. 7, lane 6).
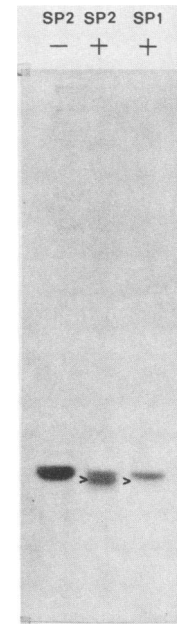

FIG. 8. SV40-directed M$_2$ protein is sensitive to protease on the cell surface. CV1 cells were infected with the SV40-M$_2$ recombinant virus, and at 48 h p.i. they were labeled with [^{35}S]cysteine and incubated in the presence (+) or absence (−) of trypsin. Samples were immunoprecipitated with antiserum to the C-terminal-region peptide (SP2) or the NH$_2$-terminal synthetic peptide (SP1). The arrows indicate the presence or absence of the trimmed M$_2$ protein (M$_2$′).

SV40-directed M$_2$ protein is transported to the cell surface. To determine if M$_2$ synthesized from the SV40 recombinant molecule could be expressed at the cell surface in the absence of synthesis of other influenza virus-specific polypeptides, the infected-cell surface was treated with trypsin. Cells were infected with SV40-M$_2$ recombinant virus, labeled with [^{35}S]cysteine, and incubated with or without trypsin. Samples were then immunoprecipitated with the SP1 or SP2 antiserum (Fig. 8). After trypsin treatment, the SP2 antiserum, which recognizes the C-terminal region of the protein, precipitated a band corresponding to normal M$_2$ and a larger amount of a polypeptide corresponding to trimmed M$_2$ (M$_2$′). The NH$_2$-terminal antipeptide serum (SP1) did not recognize the faster migratory form (M$_2$′), a result which is consistent with the NH$_2$-terminal region exposed at the cell surface. These data suggest that M$_2$ produced from the SV40-M$_2$ recombinant is transported and expressed at the cell surface in a manner similar to that in influenza virus infections.

To confirm a similar pattern of distribution of M$_2$ on the surface of the cell and its association with subcellular membranes, indirect immunofluorescence of cells transfected with SV40-M$_2$ DNA was done. CV1 cells were transfected with the SV40-M$_2$ recombinants with M$_2$ in the mRNA or vRNA sense with respect to SV40 late-region transcription and stained with affinity-purified SP1 antiserum and fluorescein-conjugated goat anti-rabbit F(ab′)$_2$ for surface fluorescence or, for permeabilized cells, with antiserum to denatured M$_2$ protein. Transfection of the SV40-M$_2$ recombinant molecules without helper virus and examination after 48 h were chosen to reduce nonspecific fluorescence due to the SV40 cytopathic effect when using lytic virus stocks. With M$_2$ DNA in the mRNA sense with respect to
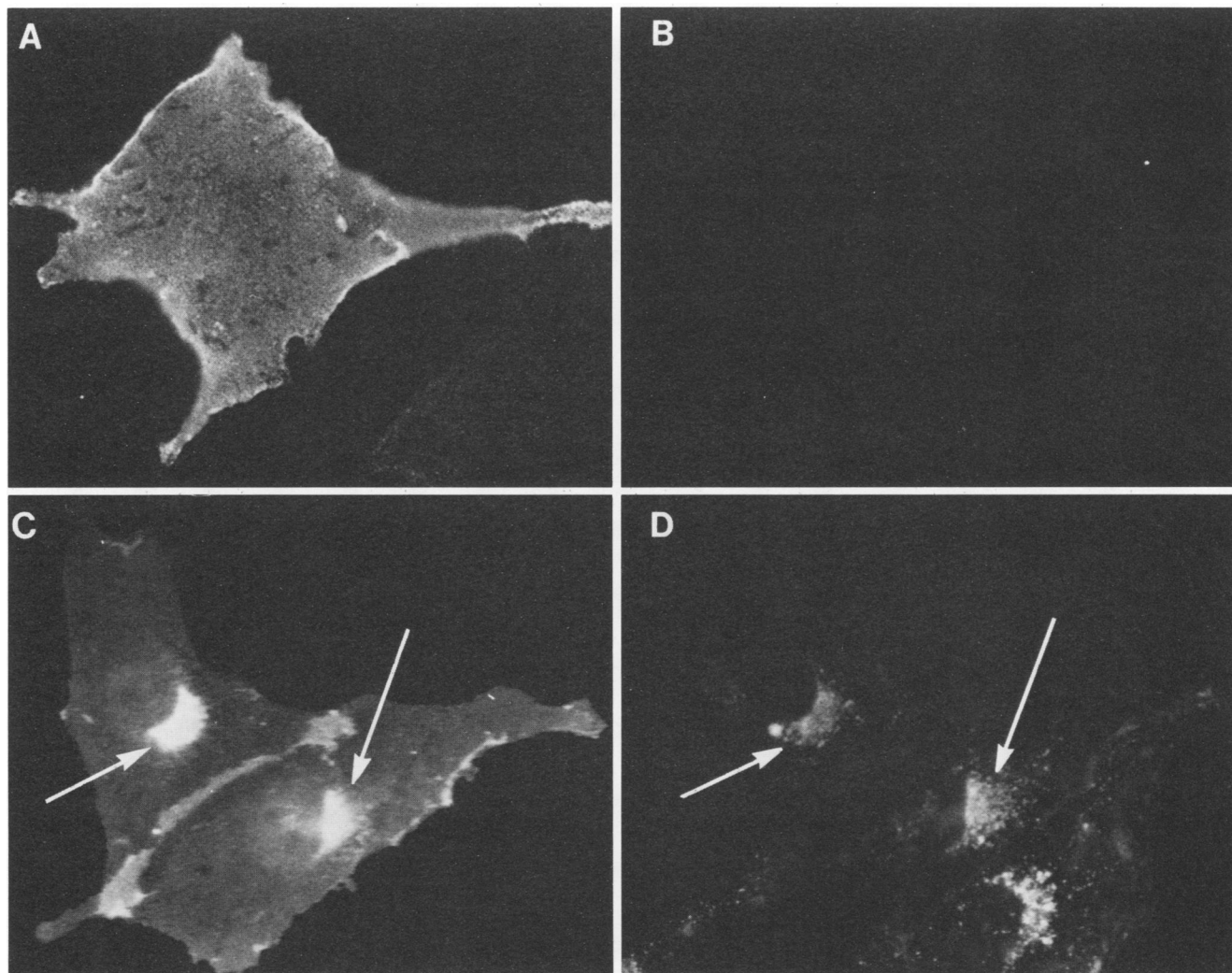
FIG. 9. Indirect immunofluorescence of cells producing M_2 from the SV40-M_2 recombinant virus. CV1 cells were transfected with SV40-M_2 DNA with M_2 in the mRNA sense (A, C, and D) or vRNA sense (B) and fixed in formaldehyde as described in Materials and Methods. The upper panels show the surface fluorescent pattern of two distinct fields after staining with affinity purified IgG to the NH_2-terminal SP1 peptide antiserum and FITC-conjugated goat anti-rabbit IgG. The lower panels show acetone-permeabilized cells incubated with IgG to the gel-purified denatured M_2 protein (DM2) and FITC-conjugated goat anti-rabbit IgG (panel C), and the same field of cells stained with rhodamine-conjugated WGA (D). The arrows indicate the Golgi apparatus. The exposure times for panels A and B were manually adjusted to be the same.

SV40 late-region transcription, a pattern of surface fluorescent staining similar to that for influenza virus infections was observed (Fig. 9A). Cells that were transfected with the SV40-M_2 molecules showed an intensity of fluorescence similar to that found for WSN-infected cells. When the M_2 DNA was in the vRNA sense, no fluorescent staining could be detected (Fig. 9B). Staining of permeabilized cells with the M_2 DNA in the mRNA sense showed intense labeling of the perinuclear Golgi apparatus (Fig. 9C), and this can be compared with rhodamine-labeled WGA staining of the same field of cells (Fig. 9D). It can be seen that in this field of cells two of the three cells were expressing the M_2 protein, and this probably reflects only the efficiency of DNA transfection. Thus, these data indicate that the M_2 protein synthesized in cells from the cloned cDNA is transported through the Golgi complex to the cell surface and inserted in the same transmembrane orientation as found in influenza virus infections.

DISCUSSION

The time course of synthesis of M_2 in WSN-infected cells indicates that it is coordinately expressed with the other influenza virus polypeptides, suggesting that the synthesis of M_2 is an integral part of the expression of the influenza virus genome. The M_2 polypeptide is encoded by a spliced mRNA derived from RNA segment 7 (20), and the kinetics of synthesis of M_2 (WSN strain) in infected CV1 cells is not detectably different from that of the M_1 polypeptide which is encoded by a colinear mRNA transcript derived from RNA segment 7. However, in other cell types (e.g., chicken embryo fibroblasts and L cells) infected with influenza A (fowl plague), virus differences in the amounts of spliced mRNA species 10, which presumably encodes M_2, have been observed (10). We have observed that among influenza A virus strains, A/Udorn/72-infected CV1 or HeLa cells apparently synthesize or accumulate less M_2 than other

strains, and this is different even from the amount of M_2 synthesized in cells infected with the closely related A/Hong/Kong/75 virus (14, 21). Thus, the control of splicing of the mRNAs in the nucleus or the ability of the M_2 mRNA to be translated requires further investigation.

We have shown previously that M_2 has properties of an integral membrane protein with 18 to 23 NH_2-terminal amino acids exposed at the infected-cell surface, and we proposed that the M_2 hydrophobic domain which minimally includes residues 25 to 43 would anchor M_2 in the membrane, leaving an approximately 54-residue COOH-terminal cytoplasmic tail (21). Our results from isolation of infected-cell microsomes show that approximately one-half of M_2 is accessible to trypsin digestion, indicating that the COOH-terminal region is not embedded in the microsomal membrane. The fragment of M_2 resistant to trypsin (apparent $M_r \cong 7,000$) was only precipitable with the NH_2-terminal SP1 antiserum and not with the SP2 antiserum, indicating that the protected fragment does not include residues 69 to 79 to which the oligopeptide SP2 antiserum was raised. The exact portion of M_2 accessible to trypsin is difficult to determine because M_2 (97 amino acids, $M_r \cong 11,000$) has an apparent electrophoretic mobility of $M_r \cong 15,000$, and this aberrant mobility may be due to the hydrophobic region which would be contained in the microsomal trypsin-resistant $M_r \cong 7,000$ fragment. From an examination of the M_2 protein sequence (see reference 21), it seems likely based on the above observations that trypsin cleavage occurs after residue 45, 49, or 53. Thus, the basic model for the insertion of M_2 into membranes is confirmed.

The association of M_2 with microsomal vesicles and accumulation of M_2 in the Golgi apparatus suggests that M_2 is transported to the cell surface by a pathway similar or identical to that of other integral membrane proteins that have been examined (reviewed in references 32 and 38). The rate of transport of M_2 occurs with a half-time of approximately 30 to 40 min, and this is similar to that observed for the rate of transport of HA to the cell surface (22). Our available evidence suggests that the M_2 potential N-linked glycosylation site (residues 20 to 23) situated on the lumenal side of microsomal vesicles is not used. This site may be unavailable to the glycosylation dolicyl-lipid enzyme complex because of its close proximity to the lipid bilayer. Although most integral membrane proteins and secreted proteins are glycosylated, it has been established that in most cases glycosylation is not a requirement for their transport through the Golgi apparatus to the cell surface (6, 7, 26, 33). Our observations that the M_2 protein expressed from a cDNA clone by the SV40 vector is transported normally and in the correct orientation to the cell surface indicate that the expression and transport of M_2 can be independent of other influenza virus-specific polypeptides, e.g., HA or NA. It remains to be determined how M_2, which contains only a single noncleavable internal hydrophobic domain, initially interacts with membranes during its synthesis, i.e., whether it is synthesized on membrane-bound polysomes and is dependent on the signal recognition particle or if it, like cytochrome b_5, associates post translationally with membranes (2, 27, 36, 37).

Previously, we proposed that the NH_2-terminal region of M_2 exposed at the cell surface had properties that matched it to the many immunological observations for a major target molecule for cross-reactive cytotoxic T cells to influenza A viruses (21). M_2 residues 1 to 10 are conserved in the predicted sequence of M_2 in the human and avian influenza strains for which the nucleotide sequence of RNA segment 7

is known (A/PR/8/34 [1, 39], A/Udorn/72 [17], A/Bangkok/1/79 [28], A/FPV/Rostock/34 [24]). We have shown here that the NH_2-terminal SP1 antiserum recognizes M_2 of all avian influenza subtypes tested (H1 to H13), suggesting that this region is antigenically conserved. Now that we have obtained an M_2 cDNA clone that can be expressed to yield M_2 at the infected-cell surface, the potential role of the NH_2-terminal region of M_2 as the target molecule on influenza virus-infected cells for cross-reactive cytotoxic T cells can be tested.

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