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Glycosylation and Surface Expression of the Influenza Virus Neuraminidase Requires the N-Terminal Hydrophobic Region

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A full-length double-stranded DNA copy of an influenza A virus N2 neuraminidase (NA) gene was cloned into the late region of pSV2330, a hybrid expression vector that includes pBR322 plasmid DNA sequences and the simian virus 40 early region and simian virus 40 late region promoters, splice sequences, and transcription termination sites. The protein encoded by the cloned wild-type NA gene was shown to be present in the cytoplasm of fixed cells and at the surface of "live" or unfixed cells by indirect immunofluorescence with N2 monoclonal antibodies. Immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of [^{35}S]methionine-labeled proteins from wild-type vector-infected cells with heterospecific N2 antibody showed that the product of the cloned NA DNA comigrated with glycosylated NA from influenza virus-infected cells, remained associated with internal membranes of cells fractionated into membrane and cytoplasmic fractions, and could form an immunoprecipitable dimer. NA enzymatic activity was detectable after simian virus 40 lysis of vector-infected cells. These properties of the product of the cloned wild-type gene were compared with those of the polypeptides produced by three deletion mutant NA DNAs that were also cloned into the late region of the pSV2330 vector. These mutants lacked 7 (*dl*k), 21 (*dl*l), or all 23 amino acids (*dl*Z) of the amino (N)-terminal variable hydrophobic region that anchors the mature wild-type NA tetrameric structure in the infected cell or influenza viral membrane. Comparison of the phenotypes of these mutants showed that this region in the NA molecule also includes sequences that control translocation of the nascent polypeptide into membrane organelles for glycosylation.

The influenza A virus neuraminidase (NA) and the hemagglutinin (HA) are membrane-anchored glycoproteins that project from the surface of virus-infected cells and from viral particles and account for strain antigenicity. The HA is of primary importance in attachment of infectious particles to the cell surface and in penetration (16, 21). The NA promotes release of budded viral particles from the cell membrane (30) and modifies carbohydrate side chains of the HA to permit its cleavage (35), an obligatory step before cell penetration.

With recombinant DNA techniques, the control of glycosylation and membrane anchorage of the influenza HA has been studied. Infection of animal cells by simian virus 40 (SV40) vectors bearing influenza HA DNA led to the production of fully glycosylated, functional HA at the cell surface (13, 15, 37). Selective amino (N)- or carboxy (C)-terminal deletions in HA DNA helped elucidate mechanisms of control of processing of the HA polypeptide. The HA has a hydrophobic signal sequence (1, 28) at its N terminus that is cleaved during entry of the nascent polypeptide into the rough endoplasmic reticulum (8). Deletion of this region resulted in failure of the nascent polypeptide to be glycosylated and loss of association with the membrane fraction of infected cells (14, 36). A second hydrophobic region, at the C terminus of the molecule, serves as a transmembrane anchorage for the fully glycosylated HA expressed at the infected cell surface and ultimately on free viral particles (44). Deletions that resulted in loss of the C-terminal hydrophobic region led to the secretion of glycosylated mutant HA polypeptide (14, 38).

Therefore, the influenza HA is like many other glycoproteins in that N- and C-terminal hydrophobic regions are separately required for glycosylation and membrane insertion, respectively (34). However, the topography of the NA polypeptide is quite different from that of the HA, suggesting that its processing would differ. Functional NA exists as a tetrameric structure comprised of disulfide bonded dimers associated by ionic interaction in pairs. The tetramer has a box-shaped head attached to a slender stalk (39). The three-dimensional structure of NA "heads" (the enzymatically active tetrameric form released from viral particles by pronase digestion) shows that each monomer is composed of six β-sheets arranged in a propeller formation and that the tetramer has fourfold symmetry. The N2 antigenic subtype NA monomer is 469 amino acids long, and for the A/Tokyo/3/67 strain, four of five potential glycosylation sites are utilized (6, 39). Sequence data in no case predict the presence of a carboxy-terminal hydrophobic region sufficient in length to span a membrane (at least 20 to 25 amino acids [7, 11, 43]). The N termini of all NA polypeptides (2, 9, 17, 25) include 12 highly conserved residues (6 hydrophilic residues followed by 6 hydrophobic residues) upstream from a variable hydrophobic region 23 amino acids in length. Therefore, there are 29 consecutive hydrophobic or neutral residues at the N terminus of the NA polypeptide. Fields and coworkers (9) suggested, based on these data, that the NA is inserted in the cell or viral membrane with an N-terminal orientation. Direct sequencing of full-size solubilized NA protein and of pronase-released NA heads suggests also that the N-terminal hydrophobic region is not processed and confirms the importance of this region for membrane anchorage (3).

To study directly the expression of the NA gene in the absence of influenza viral infection, we have cloned wild-type and selected deletion mutant NA DNAs derived from the influenza A/Udorn/72 (H3N2) NA gene in the late region of a unique SV40 expression vector. Infection of African

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green monkey kidney (AGMK) cells with this vector bearing the complete NA DNA sequence resulted in the production of a glycosylated immunoprecipitable protein associated with the membrane fraction of infected cells and detectable at the outer cell membrane by indirect immunofluorescence with monoclonal antibodies. The monomeric product of cloned NA DNA was shown to form dimers in infected cells. Neuraminidase enzyme activity in lysed vector-infected cells was detectable. The study of expression from mutant NA DNAs with deletions of sequences coding for the amino-terminal variable hydrophobic region demonstrated that mutant polypeptides fail to be translocated into membrane organelles and are not glycosylated.

MATERIALS AND METHODS

Cells and viruses. Primary AGMK cells were purchased from Microbiological Associates Bioproducts and were passaged in Eagle minimum essential medium supplemented with glutamine and 10% fetal calf serum. The concentration of fetal calf serum was reduced to 2% in minimum essential medium for maintenance of cells or during SV40 infection. Influenza A/Udorn/72 virus cloned by plaque-to-plaque passage was originally obtained from Brian Murphy. SV40 and *tsA*28, an early region temperature-sensitive mutant of SV40, were also used.

Cloning of NA DNA in pSV-2330 and preparation of the vector. The vector pSV2330 used in these investigations was derived from the vector pSV73, earlier supplied to us by P. Gruss, and an SV40 deletion mutant, *dl*-2330, previously described (19). pSV73 is a hybrid vector constructed from plasmid pBR322 and SV40 DNA after deletion of a 375-base-pair region from pBR322 that includes the *Eco*RI, *Hin*dIII, and *Bam*HI sites (Fig. 1). pSV73 has an additional deletion in the late region of the SV40 moiety between the *Hpa*II site at position 364 (converted to *Bam*HI) and the *Bam*HI site at position 2533. Therefore, pSV73 lacks SV40 late region intervening sequences. SV40 late 16S and 19S intervening sequences were added to pSV73. *dl*-2330 DNA was digested with *Kpn*I and *Bam*HI to obtain a fragment (including late mRNA intervening sequences in *dl*-2330) 358 base pairs in size extending from the *Kpn*I site at position 294 to the *Hae*II site at position 832 (converted to *Bam*HI). *dl*-2330 DNA has sustained a 180-base-pair deletion in this region, upstream from late mRNA intervening sequences. Similarly, pSV73 DNA was digested with *Kpn*I and *Bam*HI to access its *Kpn*I site. The *Kpn*I-*Bam*HI fragment from *dl*-2330 was then ligated to pSV73 at the *Kpn*I and *Bam*HI complementary ends.

Total virion RNA was reverse transcribed with a dodecamer primer (Collaborative Research, Inc., Waltham, Mass.) complementary to the 3′ common sequence in virion RNA to obtain (+) cDNA as previously described (20). After alkaline hydrolysis of template virion RNA, a second dodecamer primer (Collaborative Research), complementary to the 3′ common sequence in (+) cDNAs, was employed to prepare double-stranded influenza DNA. *Bam*HI linkers (Collaborative Research) were added to total influenza double-stranded DNA. NA DNA was selected and cloned into the *Bam*HI site of the plasmid pBR322 by standard techniques. Clones bearing a copy of the N2 NA gene were selected after screening by hybridization (20). One pBR322 clone shown to bear a complete copy of NA DNA by size and chemical sequencing at the 5′ and 3′ (+) ends was used as a source of full-length wild-type (WT) NA DNA. This NA DNA moiety was ligated into the vector pSV2330 at the *Bam*HI site in preparation of the WT expression vector and also used as a source of NA DNA in preparation of deletion mutants.

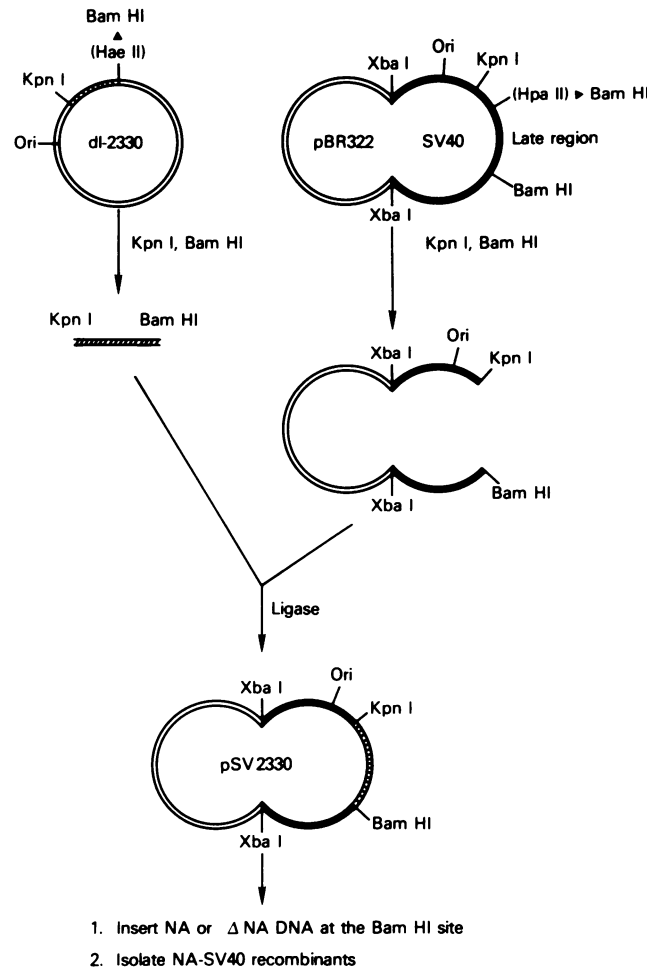

FIG. 1. Derivation of the SV40 expression vector, pSV2330. The *Kpn*I-to-*Bam*HI fragment from *dl*2330 containing SV40 late region 16S and 19S splice sequences was used to replace the analogous fragment in the vector pSV73 as described in the text.

Preparation of deletion mutant NA DNA. The technique for construction of deletion mutants is diagrammed in Fig. 2. Linear NA DNA bearing terminal *Bam*HI restriction endonuclease sites was kinased (polynucleotide kinase; P. L. Biochemicals, Milwaukee, Wis.) using [γ-^{32}P]ATP (Amersham Corp., Arlington Heights, Ill). ^{32}P-labeled molecules were made circular by ligation at *Bam*HI "sticky ends" (T4 DNA ligase; New England Biolabs, Cambridge, Mass.). Circular NA DNA was digested to completion with the restriction endonuclease *Tha*I (Bethesda Research Laboratories, Bethesda, Md.) and the free *Tha*I ends of linear molecules were digested with the exonuclease Bal 31 (Bethesda Research Laboratories). Since Bal 31 leaves single-stranded ends, these were filled in by second-strand synthesis with avian myeloblastosis virus reverse transcriptase. Blunt-ended molecules were again circularized and digested with *Bam*HI. The Bal 31 reaction was carried out for 90 s at 22°C with 1 U of enzyme.

Transfection and packaging with NA- and ΔNA-pSV2330 DNA. Recombinant vector DNA was digested to completion with *Xba*I to remove the pBR322 moiety from covalent linkage. This reduced the size of transfecting DNA to

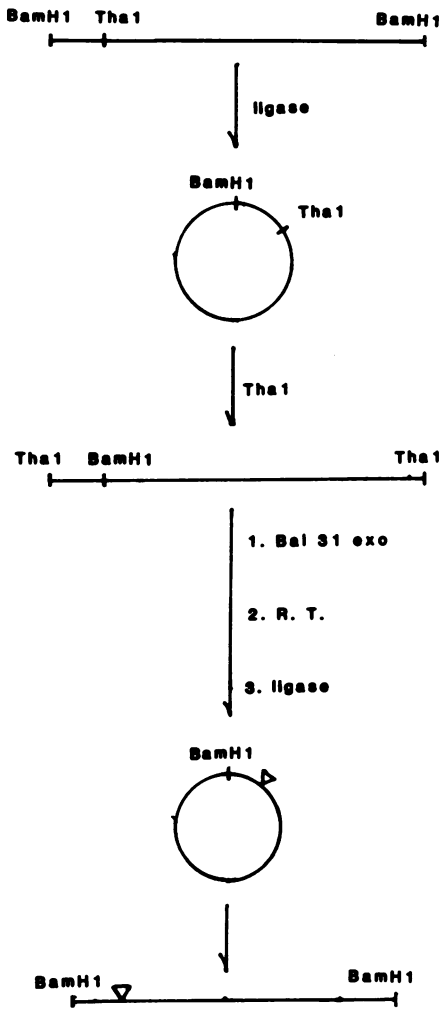

FIG. 2. Generation of deletion mutants in NA DNA. The full-length NA DNA moiety was circularized at *Bam*HI sticky ends and then made linear by digestion at a unique *Tha*I restriction endonuclease site to remove selectively the region coding for the N-terminal hydrophobic region of the NA polypeptide by digestion with the exonuclease Bal 31.

facilitate packaging into infectious viral particles. This unfractionated preparation was phenol extracted and then alcohol precipitated several times. AGMK cell monolayers in six-well plastic plates (Costar, Cambridge, Mass.) were then transfected by the diethylaminoethyl-dextran method (37) with about 2 to 5 μg of total DNA per well. After washing, cells were infected with the helper SV40 early region temperature-sensitive (*ts*) mutant, *tsA*28, at a multiplicity of infection of 0.01 to 0.1 and incubated at the nonpermissive temperature of 40°C. After 11 to 13 days of incubation, total lysis of doubly treated cells occurred. Medium from these wells was harvested and used as a source of infectious particles bearing NA or deletion mutant (ΔNA) DNA.

Radiolabeling of infected cells and analysis of polypeptides. AGMK cells were infected with NA- or ΔNA-pSV2330 preparations at a dilution of 1:10 from stock. Labeling of infected cells in the presence or absence of tunicamycin, cell lysis, immunoprecipitation of NA polypeptides, and sodium dodecyl sulfate (SDS)-polyacrylamide gel analysis were carried out as previously described (37, 38). Sheep anti-A/Udorn/72 (N2) NA serum was kindly provided by Michael Phelan.

In fractionation experiments, cells were infected and labeled as described above. After the labeling period the monolayer was treated with 0.1% trypsin in phosphate-buffered saline to remove cells from the dish. Cells were then washed once each in phosphate-buffered saline and then reticulocyte standard buffer. Cells were then suspended in reticulocyte standard buffer with 1 mM phenylmethylsulfonyl fluoride and treated with 30 strokes with a tight-fitting glass Dounce homogenizer. The postnuclear supernatant was made 0.1 M NaCl and 1 mM EDTA and then centrifuged for 40 min at 40,000 rpm in a Beckman type 50 Ti rotor. The pellet fraction (membrane) was suspended in 1 ml of RIPA buffer (37), and the supernatant fraction (cytoplasm) in reticulocyte standard buffer and phenylmethylsulfonyl fluoride was made 1× with respect to RIPA buffer and expanded by 1 to 2 volumes with RIPA buffer.

Indirect immunofluorescence. Indirect immunofluorescence was carried out with anti-N2 or anti-N1 NA monoclonal antibody preparations (42) provided as ascitic fluid by Robert Webster. AGMK cells were fixed in one of two ways. Fixing in acetone for 10 min at −70°C rendered both the outer cell and nuclear membranes permeable and caused nuclear swelling (see Fig. 4B, E, G, and H). Alternatively, cells were fixed in 3.7% formaldehyde for 10 min followed by 0.1% Triton X-100 for 5 min at room temperature, which renders the outer membrane permeable (see Fig. 4C). "Live" or unfixed vector-infected cells were counterstained with rhodamine to visualize the background of negative cells (see Fig. 4D and F).

Functional activity of NA produced from cloned full-length NA DNA. To detect NA enzyme activity, Costar T-75 flasks of AGMK cells were infected with SV40, HA-SV40 (37), or NA-pSV2330 vectors at high multiplicity (0.1 to 1.0). After adsorption, monolayers were washed repeatedly to remove fetal calf serum, and infected and uninfected cell controls were incubated for at least 72 h at 40°C in minimum essential medium supplemented only with glutamine. At this time, lysis of cells infected with SV40, HA-SV40, or NA-pSV2330 was evident by light microscopy, whereas uninfected control monolayers were intact. A separate T-75 flask of AGMK cells in monolayer was infected with influenza A/Udorn/72 virus at multiplcity of infection of 0.1 and incubated for 9 h at 37°C as a positive control, at which time the monolayer remained intact. Umbelliferone (7-hydroxycoumarin; UMF [29]), conjugated to *N*-acetylneuraminic acid (NANA), was used as the substrate for the NA. UMF is a fluorescing moiety released after hydrolysis by NA. The conjugate UMF-NANA was added to the medium in each case after the indicated incubation period to a final concentration of 1 ng/ml. Uninfected, vector-infected, or influenza virus-infected cells were then incubated with substrate for 2 h at room temperature. Fluorescence was determined with an Aminco color-fluorimeter. UMF-NANA was provided by Ron Mayner.

RESULTS

Cloning of NA and ΔNA DNA. An NA DNA insert, from a bacterial clone, that appeared to be full-length by size estimate on agarose and polyacrylamide gels and by selective restriction endonuclease digestion was sequenced at its ends and shown to include the 3'- and 5'-terminal common sequences in virion RNA (31). This full-length moiety was

used to prepare the recombinant for NA expression and deletion mutant NA DNA. For expression of WT NA, a pSV2330 clone (NA-pSV2330) bearing the complete NA moiety in (+)-sense orientation with respect to late-region SV40 promoters and other transcription signals was used as transfecting DNA for preparation of infectious NA-pSV2330 viral particles.

The complete sequence of the A/Udorn/72 (N2) NA DNA (25) had revealed that it contains a unique *Tha*I recognition sequence (5'-CGCG-3') at position 154. This site is 29 base pairs downstream from the codon for the carboxy terminal amino acid (histidine) of the hydrophobic region in the NA polypeptide, and it provided an approach to making internal deletions of sequences coding for the hydrophobic region in the NA monomer, allowing for retention of the WT initiation codon. Therefore, linear molecules were prepared with the *Tha*I site and sequences adjacent to it externalized to make them accessible to the exonuclease Bal 31 (Fig. 2). Conditions of Bal 31 digestion were then chosen to spare the NA initiation codon and adjacent downstream sequences coding for the 12 conserved N-terminal amino acids in the NA polypeptide.

Ten bacterial clones bearing ΔNA DNA were selected. The ΔNA DNA inserts were sequenced by the chemical method (27) from the 5' (+) end to determine the extent of deletion. Three of these clones (*dl*K, *dl*I, *dl*Z) were thus selected for studies of expression in AGMK cells, because the deletions fit the already stated requirements, and because sequences downstream from the deletion site were in-frame with the NA initiation codon (Fig. 3). ΔNA-pSV2330 DNAs containing (+)-sense oriented *dl*K, *dl*I, or *dl*Z sequences were used in transfection of AGMK cells to prepare ΔNA-SV2330 viral particles.

FIG. 3. 5' (+) terminal nucleotide and predicted polypeptide sequences of WT NA DNA and of deletion mutant NA DNAs, *dl*K, *dl*I, and *dl*Z. Mutagenized NA DNA was cloned in pBR322, and DNA from 10 clones was sequenced from the 5' (+) end of the insert by the chemical method. *dl*k, *dl*I, and *dl*Z DNAs were shown to bear in-frame deletions of WT NA nucleotide sequences and were therefore selected for cloning in the expression vector, pSV2330. *dl*I and *dl*Z DNAs are lacking all or nearly all sequences coding for the N-terminal hydrophobic region in NA. *dl*K is lacking sequences coding only for the seven carboxy-terminal amino acids of the hydrophobic region (residues 29 through 35). The downstream extent of the deletion on the predicted *dl*K polypeptide is eight amino acids greater than that in the *dl*I polypeptide.

Expression of NA and ΔNA mutant DNA in AGMK cells. Indirect immunofluorescence was performed on live (unfixed) or fixed AGMK cells 12 h after infection with influenza A/Udorn/72 (H3N2) virus (Fig. 4A and B) or 72 h after infection with NA- or ΔNA-pSV2330 vectors (Fig. 4C through H). Vector-infected, fixed cells contain NA antigen in the cytoplasm. In general, the fluorescence of NA antigen in fixed cells was much weaker after vector infection than after influenza viral infection of cells. However, vector-infected cells were distinctly positive in relation to controls, which included the use of anti-N1 or anti-N2 NA monoclonal antibodies against uninfected cells and against cells infected with H3N2 or H1N1 influenza A viruses (strains Udorn/72 and WSN/33, respectively). Detection of NA at the surface of intact unfixed cells was weakly positive for NA-pSV2330- and *dl*K-pSV2330-infected cells (Fig. 4D and F) and was consistently negative on unfixed cells infected with *dl*I- or *dl*Z-pSV2330 (data not shown).

The immunofluorescence assay results established that both WT and all mutant NA-vector-infected cells accumulated antigenically recognizable NA in the cytoplasm and suggested that NA- and *dl*K-pSV2330-infected cells accumulated antigen at the membrane of unfixed cells. These data do not define the intracellular localization of WT and mutant proteins. The failure to detect *dl*I and *dl*Z proteins at the cell surface suggested that these mutant NAs were defective in transport or in a processing step necessary for transport with respect to WT or *dl*K NA. To elucidate further phenotype differences among WT and mutant NA vectors, glycosylation of NA polypeptides was studied. Vector-infected cell proteins were labeled with [^{35}S]methionine in the presence or absence of tunicamycin (12, 22, 23), an inhibitor of glycosylation. When the ^{35}S-labeled proteins from total cell lysates of NA-pSV2330-infected cells were immunoprecipitated with anti-NA antibody and separated on an SDS-polyacrylamide gel, two bands were detected (Fig. 5A, lane wt [−]). The major one, a protein forming a broad band of approximately 72 kilodaltons (kd), is larger than that predicted from the amino acid sequence of the N2 NA monomer (52 kd) (25). Two findings identify the 72-kd protein as glycosylated monomeric NA. First, this band comigrates with that immunoprecipitated from influenza virus-infected cells by NA monoclonal antibody (data not shown). Second, the 72-kd band is abolished in the presence of tunicamycin and replaced by a homogenous faint band of the predicted molecular size in relation to influenza viral markers (Fig. 5A, lane wt [+]). The data of Fig. 5A also demonstrate that the level of NA polypeptide in the presence of tunicamycin is greatly reduced from that of the control, suggesting either that tunicamycin inhibits synthesis of the polypeptide in conjunction with inhibiting glycosylation or that the synthesized, unglycosylated polypeptide is degraded at an accelerated rate.

The second and minor band detected in the immunoprecipitate from NA-pSV2330-infected cells in the absence of tunicamycin is a slow-migrating protein of approximately 140 to 150 kd. We tentatively identify this band as representing the formation of NA dimer, since it comigrates with a labeled protein from virus-infected cells (Fig. 5A, lane f, band x), which is also specifically immunoprecipitated from ^{35}S-labeled influenza viral proteins (data not shown). The procedure for SDS-polyacrylamide gel electrophoresis exposes proteins to β-mercaptoethanol; therefore, the relative proportions of NA in monomeric or dimeric form in vivo cannot be estimated from these data.

The NA polypeptide coded for by *dl*K DNA exhibited a

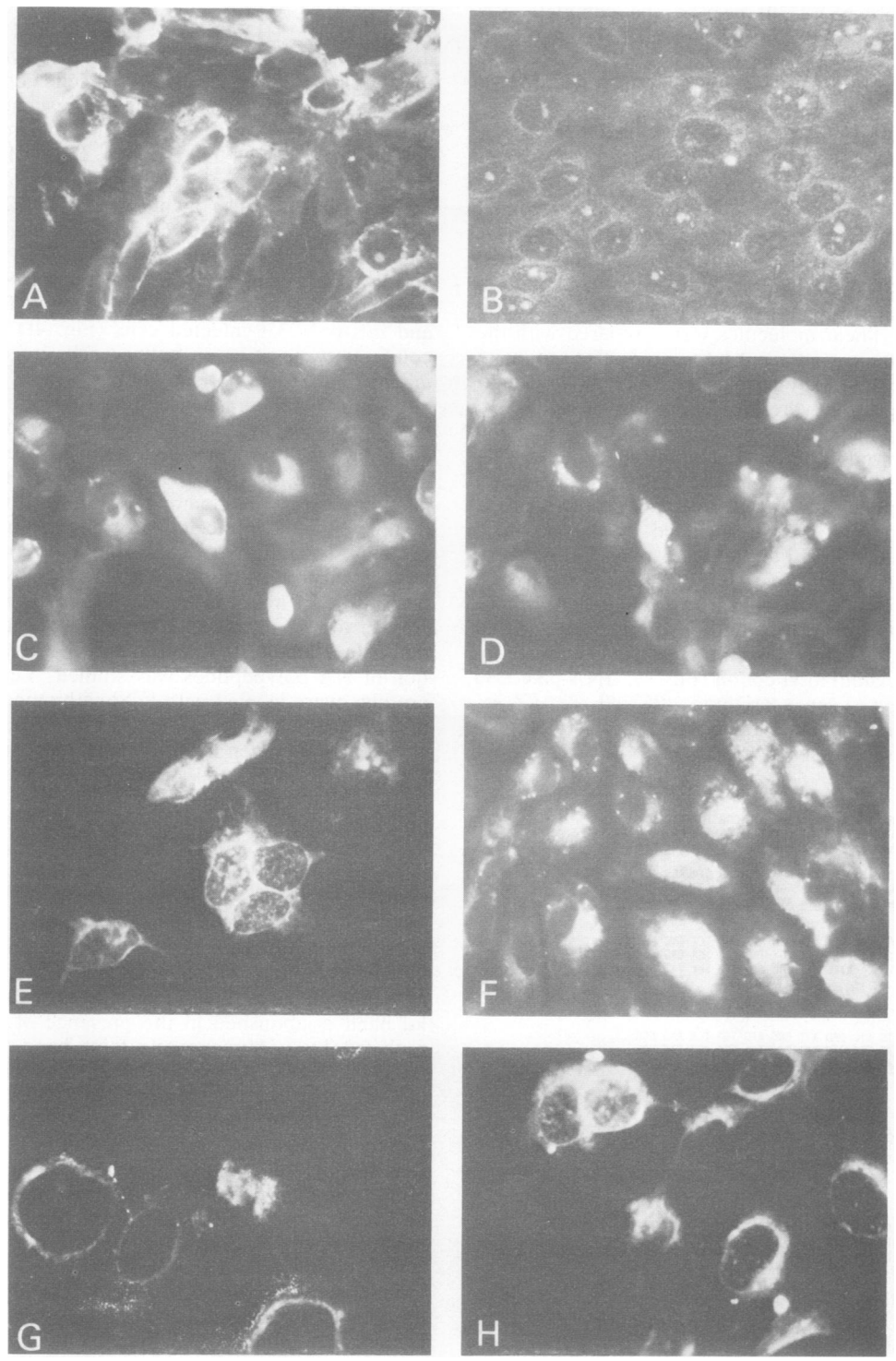

FIG. 4. Indirect immunofluorescence assay for the expression of WT or deletion mutant NA polypeptides. (A) Anti-N2 NA monoclonal antibody against influenza A/Udorn/72 (H3N2) virus-infected cells, unfixed. (B) Anti-N1 NA monoclonal antibody against A/Udorn/72 (H3N2) infected cells, fixed and permeabilized in acetone at −70°C for 10 min. (C through H) Anti-N2 NA monoclonal antibody against the following: (C) NA-pSV2330 WT vector-infected cells, fixed in formaldehyde and made permeable by treatment with Triton X-100; (D) NA-pSV2330 WT vector-infected cells, unfixed and counterstained with rhodamine; (E) *dl*K-pSV2330 vector-infected cells, fixed in acetone; (F) *dl*K-pSV2330 vector-infected cells, unfixed and counterstained with rhodamine; (G) *dl*I-pSV2330 vector-infected cells, fixed in acetone; (H) *dl*Z-pSV2330 vector-infected cells, fixed in acetone.

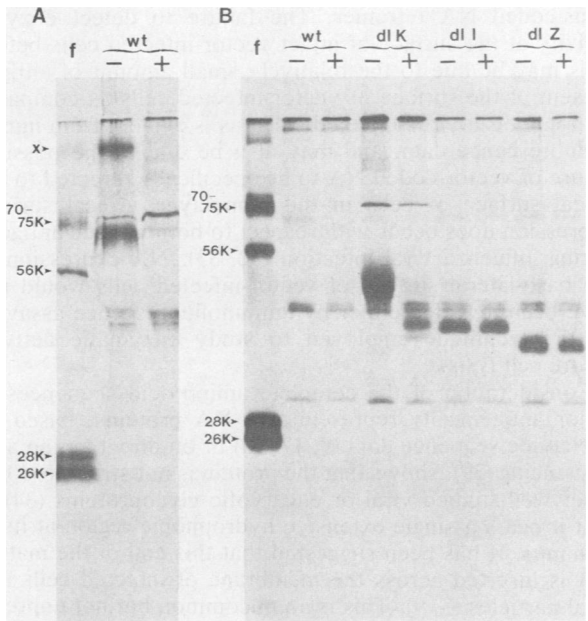

FIG. 5. Detection of immunoprecipitable NA polypeptide in lysates of NA- and ΔNA-pSV2330 vector-infected cells labeled with [^{35}S]methionine in the presence (+) or absence (−) of tunicamycin. Lysates were prepared in RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.15 M NaCl, 0.1% SDS, 0.1 M Tris [pH 7.6]) 6 h after addition of label at 100 μCi/ml. NA protein was immunoprecipitated with sheep anti-Udorn/72 (N2) NA serum and staphylococcal protein A-Sepharose beads. Immunoprecipitates were run on a 15% SDS-polyacrylamide gel at 100 V for 16 h. Lanes f contained total labeled cellular proteins from influenza A/Udorn/72 virus-infected cells labeled with [^{35}S]methionine; band x denotes a high-molecular-weight influenza virus-specific band that may represent a dimeric form of NA. (A) A 52-kd unglycosylated monomeric NA polypeptide in infected cells labeled in the presence of tunicamycin (lane wt [+]). (B) Differential effect of tunicamycin on migration rate and level of glycosylated WT and *dl*K polypeptides in contrast to unglycosylated *dl*I and *dl*Z polypeptides.

phenotype analogous to that of the WT polypeptide produced from full-length NA DNA (Fig. 5B). That is, in the absence of tunicamycin, the *dl*K polypeptide formed a broad band of 52 to 55 kd, a size greater than that predicted from the total amino acid content coded for by *dl*K DNA. A faint, slower-migrating band immunoprecipitated from the lysate of *dl*K-infected cells in the absence of tunicamycin suggests that *dl*K polypeptide may also be capable of forming a dimer. In contrast, the polypeptide isolated from cells labeled in the presence of tunicamycin bands homogeneously at a size of approximately 46 kd, which agrees with the molecular mass predicted from sequence data (Fig. 2; Fig. 5B, lane *dl*K [−]). By the criteria previously applied, *dl*K protein appears to be glycosylated. Also in parallel with results obtained with full-length NA DNA, the level of unglycosylated *dl*K protein in the presence of tunicamycin is 10- to 50-fold reduced from that of the control (estimated from band intensity).

In contrast, polypeptides produced from *dl*I and *dl*Z are not altered in size or steady-state level by tunicamycin. In each instance, the polypeptides immunoprecipitated agree in size with that predicted from the sequences (~45 and ~43 kd, respectively). This differential effect on accumulation of NA protein produced from NA, *dl*K, or *dl*Z vectors in the presence and absence of tunicamycin was confirmed by an experiment in which duplicate wells were infected, and immunoprecipitates were prepared and analyzed in parallel (data not shown). Therefore, *dl*I and *dl*Z polypeptides escape the effect of tunicamycin on the stoichiometry of the glycosylated proteins. Since tunicamycin is thought to act relatively specifically to inhibit *N*-acetylglucosamine transfer from carrier molecules to nascent glycoproteins within membrane organelles (5, 23), it seemed likely from the foregoing data and from the results of the immunofluorescence assay that *dl*I and *dl*Z polypeptides are synthesized on free ribosomes and accumulate in the cytoplasm. Such a finding would be consistent with the proposal that the N-terminal variable hydrophobic region in NA functions to mediate entry into the rough endoplasmic reticulum.

To verify the site(s) of accumulation of NA, *dl*K, and *dl*I polypeptides, infected [^{35}S]methionine-labeled cells (Fig. 6) were fractionated into membrane (M) and cytoplasmic (C) fractions as described above. By the technique employed, more than 90% of NA and *dl*K polypeptides were localized to the membrane fraction (Fig. 6A, lanes M). In contrast, most of the *dl*I polypeptide was found in the cytoplasmic fraction (Fig. 6B, *dl*K lane M and *dl*I lane C). The two fast-migrating immunoprecipitated polypeptides visualized in both M and C fractions from *dl*I-infected cells probably represent proteolytic cleavage products of the full-size *dl*I polypeptide, since trypsin was introduced to remove the vector-infected cell monolayers into suspension.

In addition to the foregoing, WT, *dl*K, *dl*I, or *dl*Z polypep-

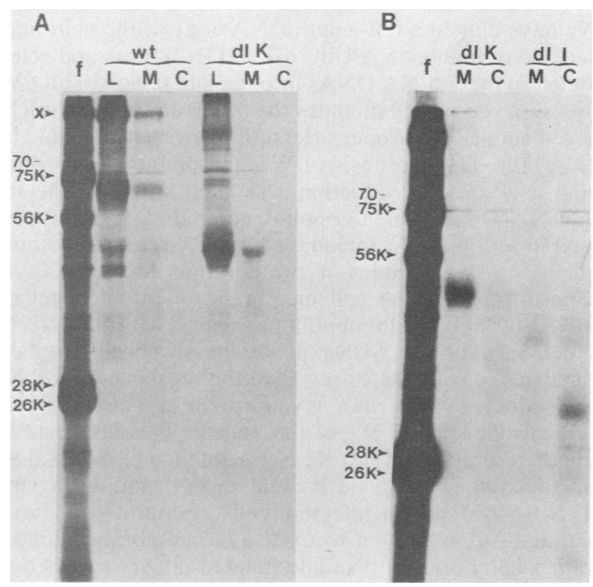

FIG. 6. Localization of WT, *dl*K, or *dl*I polypeptides to membrane (M) or cytoplasmic (C) fractions of vector-infected cells. Labeling, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis conditions were as described in the text. Cells were fractionated with a Dounce homogenizer in reticulocyte standard buffer with 1 mM phenylmethylsulfonyl fluoride. The postnuclear supernatant was made 0.1 M NaCl and 1 mM EDTA and then centrifuged at 100,000 × *g*. The resultant supernatant (C) was made 1× with respect to RIPA buffer, and the pellet (M) was suspended in RIPA buffer for immunoprecipitation. (A) WT and *dl*K polypeptides localized to membrane fraction of infected, labeled cells. Migration of the glycosylated WT or *dl*K polypeptide is not altered by fractionation procedure from that of the respective polypeptides immunoprecipitated from whole cell lysate (L) in RIPA buffer. (B) Differential localization of the glycosylated *dl*K polypeptide to the M fraction and of the unglycosylated *dl*I polypeptide to the C fraction of vector-infected cells. Lanes f contained [^{35}S]methionine-labeled influenza A/Udorn/72 viral proteins.

tides were not detected as secretory products in the medium from infected cells, with techniques that readily resulted in detection of secreted HA deletion mutant polypeptides (38) (data not shown).

Detection of enzymatic activity of the product of the cloned WT NA gene. Cleavage of UMF from its conjugate with NANA was detected at low levels by fluorescence of free UMF after viral lysis of NA-SV40 cells had occurred as described above. The reaction reached its limit after about 2 h of incubation at room temperature, independent of the concentration of UMF above 1 ng/ml of medium. Enzyme activity was not detected on the NA vector-infected cell monolayer at any time before viral lysis of cells. Under these same conditions of cell lysis, cleavage of substrate was not detected when cells were infected with WT SV40 or HA-SV40 (37). Uninfected cells served as an additional negative control, and unlysed influenza A/Udorn/72 virus infected cells 9 h after adsorption of infecting virus served as a positive control. The presence of fetal calf serum in the medium after NA vector infection abolished all detectable enzyme action on the UMF-NANA substrate. A 75-cm^2 monolayer of NA vector-lysed cells manifested enzyme activity sufficient to produce a change in fluorescence intensity of 0.2 units. For comparison, an unlysed 75-cm^2 monolayer of influenza virus-infected cells produced a 10-unit change in fluorescence intensity.

DISCUSSION

We have cloned a full-length DNA copy of the neuraminidase gene of influenza A/Udorn/72 (H3N2) virus and selected deletion mutant NA DNAs into the late region of an SV40 expression vector that includes the late promoter and mRNA splice sequences in proper orientation to produce stable late mRNA (19). The synthesis of WT and mutant NA proteins during a lytic SV40 infection was then studied. The full-length NA DNA, which contains noncoding regions represented in virion RNA, including 5′ and 3′ terminal common sequences (31), encodes a protein that localizes in the cytoplasm and on the cell membrane of infected cells as detected by indirect immunofluorescence with anti-N2 NA monoclonal antibody. SDS-polyacrylamide gel electrophoretic analysis of cell lysates revealed the presence in WT NA-vector-infected cells of a glycoprotein, precipitable with heterospecific anti-N2 NA serum, that appeared to represent a complete product of the N2 NA sequence (25). NA dimer molecules appeared to be present in both influenza virus- and NA-SV40 vector-infected cells. Naturally occurring functional NA is known to exist as a membrane-anchored tetramer composed of disulfide bonded dimers that become ionically associated in pairs (39). The tetrameric form of the molecule is essential for enzyme activity (6). The detection of an NA dimer in vector-infected cells, in addition to the observation of membrane fluorescence of infected unfixed cells, suggested that the product of the cloned full-length NA DNA might be capable of forming the enzymatically active tetramer. The function of the product of the cloned NA DNA was confirmed by detection of activity when lysed NA-pSV2330-infected cells were incubated with a conjugate of UMF to NANA. UMF-NANA is a low-molecular-weight substrate for the neuraminidase and should gain access to an enzymatic active site with a minimum of steric hindrance. This situation is in contrast to the natural one, in which the NA substrate is normally a cellular glycoprotein or the influenza viral HA. Therefore, we do not necessarily conclude that the weak enzymatic activity observed demonstrates the formation of a complex identical to the influenza virus-coded NA tetramer. The failure to detect enzyme activity at the surface of intact vector-infected cells before lysis may be due to the relatively small amount of antigen present at the surface of vector-infected cells as compared with influenza virus-infected cells, as is evident from immunofluorescence data, and may also be due to the possible failure of vector-coded NA to be specifically directed to the apical surface of cells in the monolayer. Apical surface expression does occur with respect to both surface antigens during influenza viral infection (32, 33). NA expression at the baso-lateral surface of vector-infected cells would not have been detected either by immunofluorescence assay or by the technique employed to study enzymatic activity before cell lysis.

Consideration of the complete amino acid sequences of major antigenically representative NA proteins, based on nucleotide sequence data (9, 17, 25) or on direct amino acid sequencing (39), shows that the protein is not structured like other well-studied viral or eucaryotic glycoproteins (34) in that it bears a single extensive hydrophobic region at its N terminus. It has been suggested that this end of the mature NA is inserted across the membrane of infected cells and viral particles (3, 9). This is an uncommon but not unprecedented orientation for membrane proteins, as a similar one has been proposed for isomaltase (4, 10) and intestinal brush border aminopeptidase (26). A comparison of the determined amino acid sequences of solubilized NA and of NA tetrameric heads suggested that the N terminus is not truncated during processing (3). We studied the mechanisms affecting glycosylation and membrane insertion of the NA protein by assessing the phenotype of polypeptides coded for by NA DNA that had sustained in-frame deletions of part (*dl*K) or all (*dl*I, *dl*Z) of the coding region for the hydrophobic N terminus.

*dl*K retained all but the seven carboxy-terminal amino acids of the hydrophobic region. In addition, the deletion in *dl*K extended 52 amino acids downstream from the hydrophobic region. However, the *dl*K polypeptide was glycosylated and was detected at the cell membrane by immunofluorescence assay on unfixed infected cells. Cell fractionation studies demonstrated that *dl*K accumulated in the membrane fraction. In the presence of tunicamycin, *dl*K was not glycosylated, and its accumulation was greatly diminished. In these ways, the *dl*K polypeptide was not distinguishable from that of wild-type NA DNA. In contrast, *dl*I and *dl*Z polypeptides were not detected at the cell surface and were not glycosylated, and their level was not detectably reduced by tunicamycin. *dl*I polypeptide was detected predominantly in the cytoplasmic fraction of infected cells. Although *dl*I lacked all but the initial three amino acids of the variable hydrophobic region in the WT NA protein, the carboxy-terminal extent of the deletion in *dl*I was eight amino acids shorter than that in *dl*K. Therefore, the observed differences in phenotype between *dl*K and *dl*I are not likely to be related to the deletion of sequences downstream from the N-terminal hydrophobic region.

We conclude that the hydrophobic region at the N terminus of the NA protein includes a sequence that directs the translocation of the nascent NA polypeptide into the rough endoplasmic reticulum, which leads to glycosylation and cell surface expression. This is one functional definition of a signal sequence (1, 28). If the N terminus were only a transmembrane anchor, we would expect the phenotype of mutants *dl*I and *dl*Z to be similar to that of HA C-terminal deletion mutants, which are glycosylated and secreted (14, 38). We have not shown, however, that the N terminus of the

NA fulfills a more rigorous definition of a signal sequence, that it mediates a translational arrest in the presence of a signal recognition particle, and that deletion of hydrophobic sequences eliminates the translational arrest (40–41b). Since the NA protein is not cleaved, a clear understanding of whether or how its N terminus interacts with the signal recognition particle during and after translation would be of great interest. Additionally, our data do not rule out the possibility that the altered phenotype of mutant NA polypeptides *dl*I and *dl*Z is the result of a conformational change randomly induced by the sequence deletions. If so, they do demonstrate at least a conformational requirement for the deleted hydrophobic sequences.

If a signal sequence or an analog to one exists at the N terminus of the NA, it is likely to include the initial six conserved hydrophobic residues in the WT sequence which were not deleted from mutant polypeptides (Fig. 3). Characteristic of a signal sequence (5, 18, 24), these residues are immediately downstream from the hydrophilic N-terminal tail of the molecule. Sequence data suggests that a signal sequence needs to be at least 12 amino acids in length, whereas about 20 to 25 amino acids are necessary for transmembrane anchorage (10, 11, 43). If the signal sequence in the NA begins at Lys at position 6, it is interrupted after only 10 hydrophobic residues in *dl*I and after 7 in *dl*Z. In contrast, the hydrophobic region in *dl*K polypeptide was shortened to 22 amino acids, and the protein appears to be glycosylated and inserted in the outer cell membrane with an efficiency equivalent to that of the wild type. It might be possible to distinguish the location and extent of the sequence crucial to translocation from that necessary for membrane insertion, for example, by extending the deletion in *dl*K DNA gradually upstream, progressively shortening the extent of the hydrophobic region, but preserving a length sufficient to function in translocation. Ultimately, one might observe secretion of a glycosylated mutant NA protein analogous to HA mutant proteins that lack C-terminal hydrophobic sequences (14, 38).

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