MOLECULAR AND CELLULAR BIOLOGY, Dec. 1986, p. 4317–4328
0270-7306/86/124317-12$02.00/0

Vol. 6, No. 12

Determination of the Orientation of an Integral Membrane Protein and Sites of Glycosylation by Oligonucleotide-Directed Mutagenesis: Influenza B Virus NB Glycoprotein Lacks a Cleavable Signal Sequence and Has an Extracellular NH_2-Terminal Region

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Received 23 June 1986/Accepted 13 August 1986

The membrane orientation of the NB protein of influenza B virus, a small (M_r, ~18,000) glycoprotein with a single internal hydrophobic domain, was investigated by biochemical and genetic means. Cell fractionation and protein solubility studies indicate NB is an integral membrane protein, and NB has been shown to be a dimer under nonreducing conditions. Treatment of infected-cell surfaces with proteinase K and endoglycosidase F and immunoprecipitation with a site-specific antibody suggests that the 18-amino-acid NH_2-terminal region of NB is exposed at the cell surface. Oligonucleotide-directed mutagenesis to eliminate each of the four potential sites of N-linked glycosylation and expression of the mutant NB proteins in eucaryotic cells suggest that the two sites adjacent to the NH_2 terminus are glycosylated. This provides further evidence that NB, which lacks a cleavable NH_2-terminal signal sequence, has an exposed NH_2 terminus at the cell surface.

In eucaryotic cells, glycoproteins are either translocated completely across the membrane into the lumen of the endoplasmic reticulum for soluble proteins destined for secretion or they are asymmetrically integrated into distinct cellular membranes. Integral membrane proteins may span the membrane once or more with the NH_2 and COOH termini of the polypeptide chain on either side of the bilayer. These proteins are anchored in membranes by a membrane-spanning domain which is generally found to consist of 20 or more hydrophobic or uncharged amino acids. It has been established that the orientation of an integral membrane protein is absolute and is maintained regardless of the final destination of the protein (e.g., in the plasma membrane) (reviewed in references 3 and 54). Similarly, the addition of asparagine N-linked carbohydrate to an integral membrane protein is also asymmetric and occurs in the lumen of endoplasmic reticulum vesicles at the amino acid sequence Asn-X-Ser/Thr (reviewed in reference 17). However, determination of the transmembrane orientation and location of the precise sites used for addition of carbohydrate by peptide analysis are often difficult.

We have been interested in examining properties of a glycoprotein, NB, of influenza B virus which circumstantial evidence indicated was an integral membrane protein. In the course of these studies we developed a method, based on site-specific mutagenesis and expression of mutant cDNAs in eucaryotic cells, that enabled us to determine the orientation of the protein in membranes and the precise sites of carbohydrate addition.

Influenza B virus NB glycoprotein (100 amino acids) is translated from a bicistronic mRNA derived from RNA segment 6 that also encodes the neuraminidase (NA) glycoprotein by using overlapping reading frames (28, 48). The first AUG codon in the mRNA is used for the initiation of NB and the second AUG codon, separated from the first AUG codon by four nucleotides, is used to initiate NA. NB (M_r, ~18,000) has been shown to be abundantly expressed in influenza B virus-infected cells and to be glycosylated based on incorporation of [^{3}H]glucosamine and a shift in apparent molecular weight after treatment with endoglycosidase H (endo H) from M_r ~18,000 to ~11,500 (48). Examination of the predicted amino acid sequence of NB (Fig. 1; data derived from references 48 and 49) indicates that it contains a region of 22 uncharged amino acids (residues 19 to 40). This region has a hydropathic index of >20, a value normally found for regions of proteins that interact with membranes (20), but biochemical evidence that NB is an integral membrane has not been reported and the subcellular localization of NB has not been determined. NB contains four potential sites (Asn-X-Ser/Thr) for the addition of N-linked carbohydrate, two on each side of the hydrophobic domain. As discussed above, the actual sites used depend on the orientation of the protein in membranes. We present both biochemical and molecular-genetic evidence that NB is an integral membrane protein expressed at the cell surface with an NH_2-terminal region and two carbohydrate chains exposed extracellularly, an orientation unusual for a protein lacking a cleavable signal sequence.

MATERIALS AND METHODS

Viruses and cells. Influenza B virus (B/Lee/40) was grown in embryonated eggs. CV1 and MDCK cells were passaged and infected as described (22, 25).

Antibody production. Denatured NB was isolated from MDCK-infected cells by preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. NB was eluted from the gels and used to immunize rabbits as described previously (29). An antisera to a peptide synthesized to NB residues 58 to 72, kindly provided by C. D. Richardson (National Institutes of Health), was prepared as described previously (43).

Isotopic labeling of infected-cell lysates, immunoprecipitation, and gel electrophoresis. Influenza B virus-infected CV1 or MDCK cells were labeled from 7 to 9 h postinfection (p.i.)

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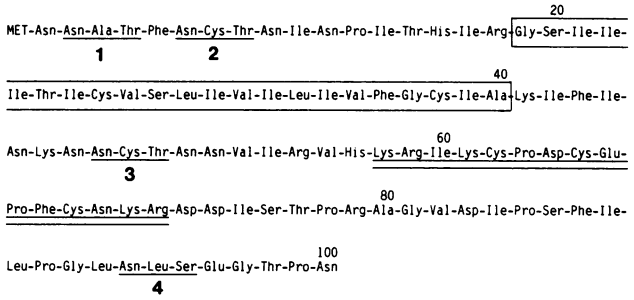

FIG. 1. Predicted amino acid sequence of B/Lee/40 NB. The amino acid sequence of NB predicted from the nucleotide sequence of the NA/NB mRNA (data derived from reference 48). The four potential glycosylation sites are underlined and numbered with respect to their position from the NH_2 terminus, the long uncharged hydrophobic domain is boxed, and the oligopeptide used to make an antibody to NB is indicated by double underlines.

with 50 to 200 μCi of [^{35}S]cysteine in Dulbecco modified Eagle medium (DME) deficient in cysteine. CV1 cells infected with simian virus 40 (SV40) recombinant viruses were labeled at 40 to 48 h p.i. for 3 h with 100 μCi of [^{35}S]cysteine in DME deficient in cysteine. Both immunoprecipitation and analysis by polyacrylamide gel electrophoresis were carried out as described previously (25).

Cell fractionation, surface proteolysis, surface endo F treatment, and microsome isolation. Cell fractionation and solubilization of integral membrane proteins were done as described previously (12, 29). Treatment of intact cells with 100 μg of proteinase K per ml was done essentially as described previously (29) except that after [^{35}S]cysteine labeling of infected cells, the medium was replaced with DME for 1 h to allow for transport of the labeled protein. To remove carbohydrate residues from the cell surface, intact cells were treated with endo F (Boehringer Mannheim Biochemicals). [^{35}S]cysteine-labeled B/Lee/40-infected MDCK cells were scraped from tissue culture dishes (2 by 60 mm) in 2 ml of phosphate-buffered saline and divided into two aliquots. One unit of endo F was added to one aliquot, and both were incubated at 37°C for 3 h with occasional mixing. Cells were washed four times with 5 ml of ice-cold phosphate-buffered saline by centrifugation (1,000 × *g*, 5 min) to remove the enzyme and then lysed and immunoprecipitated as described earlier (25). A crude preparation of microsomes was made from infected cells and treated with trypsin as described by Zebedee et al. (56).

Endoglycosidase treatment of immunoprecipitated proteins. [^{35}S]cysteine-labeled influenza B/Lee/40-infected CV1 cells were lysed and immunoprecipitated (25) by using antisera to NB or NA (a generous gift of R. G. Webster). Protein A–agarose-antibody-antigen complexes were boiled for 4 min in 60 μl of 50 mM Tris hydrochloride (pH 7.4)–0.5% SDS, and the supernatant was diluted with an equal volume of 0.1 M sodium citrate (pH 5.3)–1 mM phenylmethylsulfonyl fluoride and 2 mU of endo H (Miles Laboratories, Inc.) or 80 mU of endo F and incubated at 37°C for 16 h. The reaction was terminated by the addition of an equal volume of gel sample buffer (25), and samples were analyzed by gel electrophoresis.

Construction of SV40-NB-NA recombinant viruses and expression in CV1 cells. Plasmid pBNA1 (49) contains a full-length cDNA copy of influenza B virus RNA segment 6 (B/NB/NA DNA) cloned by G/C tailing into the *Pst*I site of pBR322. The B/NB/NA DNA was released by *Pst*I digestion, and the fragment was isolated on an agarose gel. The G/C tails were removed by controlled digestions with nuclease *Bal* 31, the DNA molecules were blunt-ended with T4 DNA polymerase, and *Xho*I linkers were added. The resulting DNAs were cloned into the *Xho*I site of a plasmid (pSV93) which contains an SV40 late-region replacement vector. pSV93 was adapted from pSV-2330 (34) and is very similar to pSV103 already described (38), except that in pSV93 the *Xho*I cloning site in the late region is flanked by *Bam*HI sites. The 5′ end points (mRNA sense) of the nuclease *Bal* 31 deletions were determined by nucleotide sequencing. The ~600-nucleotide *Bam*HI fragment that contained the 5′-end deletions from those clones considered suitable for further analysis were reconstructed into a pSV93 vector plasmid that contained the large 3′-end *Bam*HI fragment of B/NB/NA DNA. The resulting plasmids contained deletions at the 5′ end of the B/NB/NA DNA in the 5′-untranslated region but retained the original 3′ end of B/NB/NA DNA, including a G/C tail. Another clone, pSV93BNAΔ39, was produced by restriction enzyme cleavage of pBNA1 at the *Hae*III sites at viral nucleotides 39 and 1057 and the subsequent addition of *Xho*I linkers and reconstruction of the correct 3′ end of the clone in pSV93 as described above.

Two of the B/NB/NA clones containing untranslated leader region deletions were used in the experiments described here. pSV93BNAΔ4 contains a deletion of three influenza virus-specific nucleotides from the 5′ end of the DNA in the mRNA sense, and pSV93BNAΔ39 contains a deletion of 38 influenza virus-specific nucleotides.

For transfection, the vector DNAs were released from the bacterial plasmid sequences by *Sac*I digestion, and the DNAs were circularized with T4 DNA ligase. Four micrograms each of total *Sac*I-digested DNA and an SV40 early-region deletion mutant (*dl*1055) to act as helper virus (40) were introduced into CV1 cells by DEAE-dextran-mediated transfection (30). Virus stocks were then produced as described earlier (26). All DNA manipulations were done as described previously (33).

Site-specific mutagenesis of potential N-linked glycosylation sites in NB. To facilitate site-specific mutagenesis of the Asn residue to a Ser in the potential N-linked carbohydrate addition sites of NB, a 1,386-base-pair (bp) *Kpn*I-to-*Xba*I fragment was isolated from pSV93BNAΔ4. The *Kpn*I-*Xba*I fragment contains 355 bp of SV40 late-region sequence in addition to 1,031 nucleotides from the 5′ end of B/NB/NA DNA, including the entire NB coding region. The *Kpn*I-*Xba*I fragment was cloned into the *Kpn*I and *Xba*I sites situated in the polylinker of the replicative form of bacteriophage M13mp19 to yield M13mp19K-X. Site-specific mutagenesis was done by using the protocol of Zoller and Smith (57) and mismatch oligonucleotides. The specific nucleotides and resulting amino acids that were changed in NB are described below (see also Fig. 7). Briefly, the 18- to 19-nucleotide mutagenic oligonucleotide containing a 1- or 2-bp mismatch was annealed to M13mp19K-X single-stranded DNA together with the 15-mer standard M13 sequencing primer oligonucleotide. The primers were elongated to form double-stranded circular DNA by adding deoxynucleotide triphosphates, the Klenow fragment of *Escherichia coli* DNA polymerase I, and T4 DNA ligase. The resulting DNA was then used to transfect *E. coli* TG1, and an appropriate dilution was plated out onto agar with freshly grown *E. coli* TG1. Plaques (phage) were directly transferred to nitrocellulose filters, and the fixed DNA was hybridized with ^{32}P-5′-end-labeled mutagenic oligonucleotide. The filters were

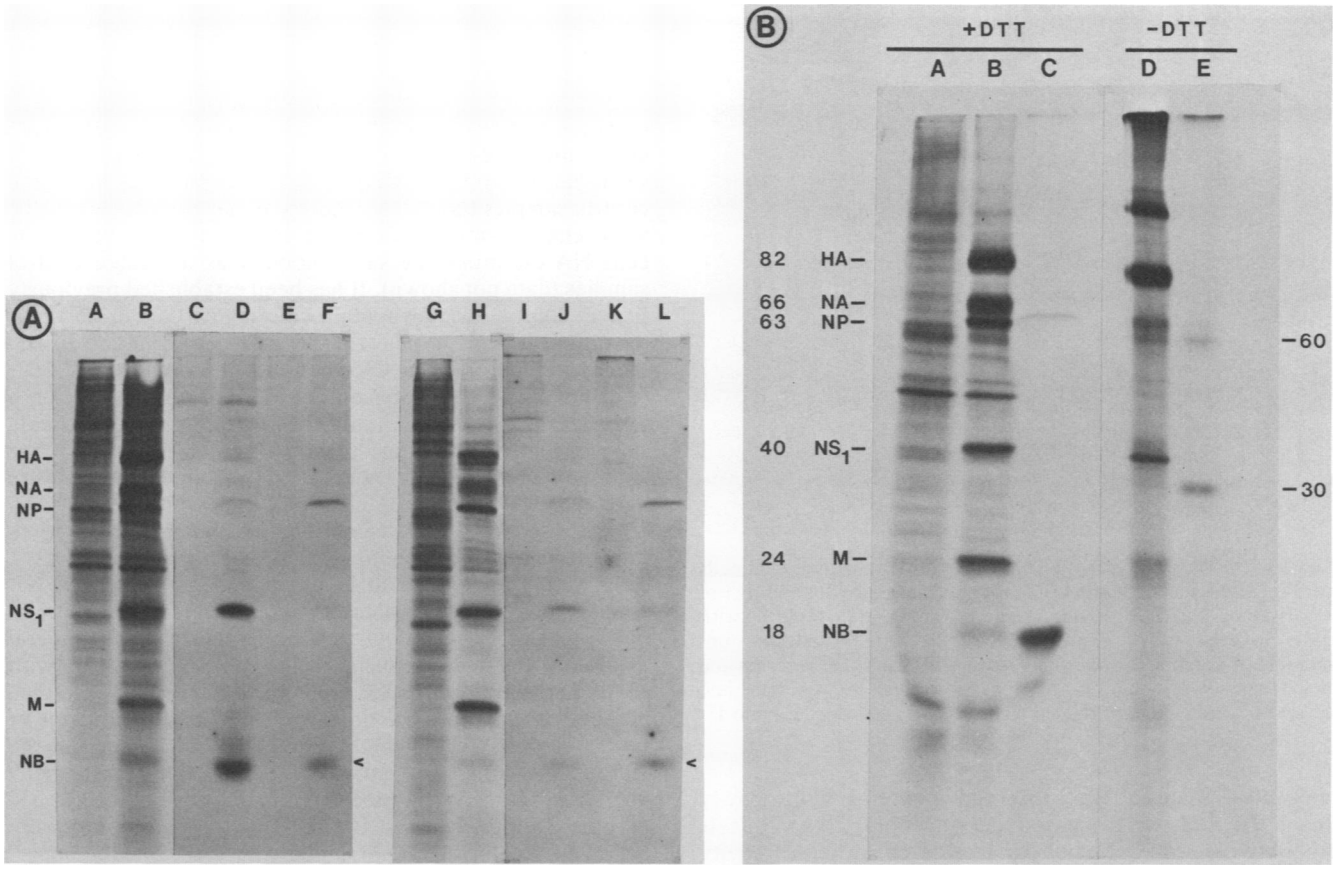

FIG. 2. (A) Production of antisera to NB. Influenza B/Lee/40-infected CV1 (lanes A to F) and MDCK (lanes G to L) cells were labeled with 50 μCi of [^{35}S]cysteine at 8 h p.i. for 2 h. Immunoprecipitations were done as described earlier (25). Lanes: A and G, mock-infected cells; B and H, infected cells; C and I, immunoprecipitation of mock-infected-cell lysates with DNB antiserum; D and J, immunoprecipitation of infected cell lysates with DNB antiserum; E and K, immunoprecipitation of mock-infected-cell lysates with CNB antiserum; F and L, immunoprecipitation of infected-cell lysates with CNB antiserum. Molecular weight markers for all polyacrylamide gels are influenza B/Lee/40 infected-cell polypeptides of the following molecular weights: HA, 82,000; NA, 66,000; NP, 63,000; NS, 40,000; M, 24,000; NB, 18,000. (B) NB is a disulfide-bonded multimer in infected cells. CV1 cells were infected with B/Lee/40, labeled with [^{35}S]cysteine, and either analyzed as direct lysates or immunoprecipitated with DNB antibody. Lanes: A, B, and C, dithiothreitol treated before electrophoresis (+DTT); A, mock-infected cells; B, infected cells; C, lysate from lane B immunoprecipitated with DNB antibody; D and E, subjected to electrophoresis in the absence of reducing agent (−DTT); D, direct lysate of infected cells; E, lysate from lane D immunoprecipitated with DNB antibody. The apparent molecular weights (×1,000) of influenza B/Lee/40 polypeptides are shown to the left of the figure, and the deduced molecular weights (×1,000) of the NB multimers are noted on the right.

washed at increasing stringency until only a subset of phage retained hybridized oligonucleotide. The presumptive mutants were plaque purified, and their authenticity was verified by obtaining the nucleotide sequence of the phage DNA in the region of mutagenesis by dideoxy chain termination (45). The replicative form of the mutant phage was purified by CsCl equilibrium density centrifugation, and the *Kpn*I-*Xba*I 1,386-bp fragment was isolated and recloned into pSV93BNAΔ4, replacing the wild-type *Kpn*I-*Xba*I 1,386-bp fragment. To further verify that the appropriate mutants had been obtained, the nucleotide sequence of the NB coding region was obtained of the reconstructed pSV93BNA mutant plasmids. This was done by direct dideoxynucleotide sequencing of CsCl-purified and alkaline-denatured plasmid DNA (5). The DNAs were then used for transfection as described above.

RESULTS

Production of antisera specific to NB. To facilitate experiments and to confirm indirectly a portion of the sequence of NB, two antisera were produced that are specific for NB. The predicted amino acid sequence of NB (Fig. 1; data derived from references 48 and 49) was used to synthesize an oligopeptide from a COOH-terminal region (CNB; residues 58 to 72). Rabbit site-specific antisera were prepared to the oligopeptide coupled to keyhole limpet hemocyanin. The second antisera (DNB) was produced in rabbits to gel-purified and denatured NB. The specificity of the two antisera for NB was tested by immunoprecipitation of both [^{35}S]cysteine-labeled influenza B/Lee/40 virus-infected CV1 and MDCK cell extracts and is shown in Fig. 2A. It can be seen that the DNB and CNB antisera readily precipitated NB (lanes D and F, J and L, respectively) and that these antisera did not precipitate any polypeptides from uninfected cells of similar mobilities to NB. In samples immunoprecipitated with the DNB and CNB antisera, bands of mobility similar to that of NS_1 and NP were also observed. This precipitation is probably artifactual and does not affect interpretation of the data presented here. In the immunoprecipitates from infected CV1 cell lysates, a region of diffuse

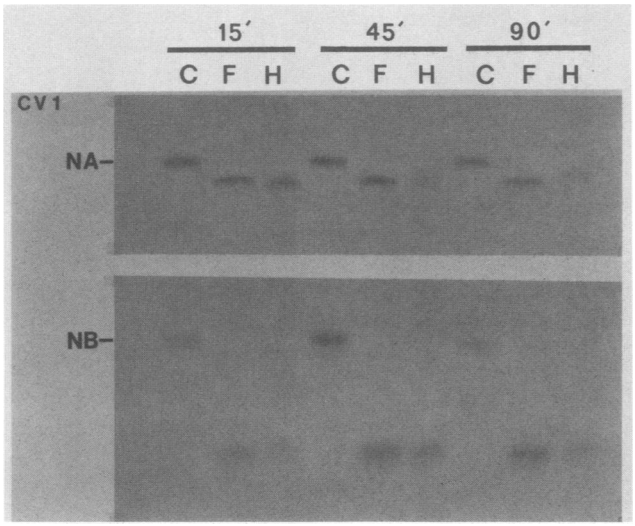

FIG. 3. NB carbohydrate chains are endo H sensitive. CV1 cells were infected with influenza B/Lee/40 and labeled with 100 μCi of [^{35}S]cysteine 7 h p.i. for 20 min. The medium was then replaced with DME for the times shown. The cells were lysed and immunoprecipitated with either anti-Na serum or anti-DNB serum and then treated with endo F or H as described in Materials and Methods. Lanes: C, control, no treatment; F, endo F; H, endo H.

radioactive material was observed migrating more slowly than NB, and, as discussed later (see Fig. 8A), this is probably caused by carbohydrate side chain heterogeneity on NB such as extensions on the N-linked glycans. In the immunoprecipitates from infected MDCK cells, a region of diffuse radioactive material can be seen migrating between NP and NS_1 (Fig. 2A, lanes J and L), and this is discussed below (see Fig. 5).

NB is a disulfide-linked dimer. NB contains seven cysteine residues (Fig. 1), and, if all possible intramolecular disulfide bonds are formed, one cysteine residue per NB molecule is available for intermolecular disulfide bonding. [^{35}S]cysteine-labeled NB was immunoprecipitated with DNB antisera, and the proteins were subjected to electrophoresis in the absence of reducing agent. A major band of M_r ~30,000 and a lesser band of M_r ~60,000 were observed, but no band was observed at M_r ~18,000 (reduced form) (Fig. 2B). These data suggest that NB is a dimer of identical chains and may form tetramers. However, it cannot be ruled out that the tetramers may be formed by a hydrostatic interaction of dimers rather than by disulfide bonding. The stoichiometry of dimers to tetramers (Fig. 2B) suggests that the tetramers could be formed during the isolation procedure. The apparent differences in molecular size between monomers and dimers may reflect conformational differences due to intramolecular and intermolecular disulfide bonding or aberrant binding of SDS.

Carbohydrate complexity of NB. Glycoproteins acquire high-mannose sugars in the lumen of the rough endoplasmic reticulum and, after transport of vesicles to the Golgi apparatus, are often processed to form complex sugars (reviewed in reference 17) that, unlike high-mannose sugars, are resistant to digestion by endo H (52). To examine whether the carbohydrate on NB is processed to complex carbohydrate, influenza B virus-infected CV1 or MDCK cells were labeled for 15 min with [^{35}S]cysteine and then incubated in DME for various periods. Cell lysates were immunoprecipitated with either DNB antibody or antibody to NA (as a control) and then digested with endo H. Another aliquot of the samples was treated with endo F, an enzyme which digests N-linked carbohydrate regardless of its forms (6). NA immunoprecipitated from CV1 cells acquired endo H resistance with a half-time of ~45 min (Fig. 3, lanes H). It can be seen that NA did not attain the same mobility as untreated control samples (lanes C), suggesting that one or more of the sugar chains remains in the high-mannose form. In MDCK cells NA did attain the same mobility as untreated control samples (data not shown). It has been established previously that glycoproteins may contain differing carbohydrate forms in different cell types (7, 36). In contrast, NB immunoprecipitated from CV1 cells (Fig. 3) or MDCK cells (data not shown) did not acquire endo H resistance in a 2.5-h chase period (up to 90 min is shown in Fig. 3). These data suggest that NB contains carbohydrate side chains of the high-mannose type that are not processed to complex sugars in either MDCK or CV1 cells.

NB is associated with membranes and has properties of an integral membrane protein. Influenza B virus-infected CV1 cells were fractionated to examine the association of NB with membranes by a standard procedure (12) used previously to show the association of influenza virus integral membrane proteins, hemagglutinin (HA), NA, and M_2 with cellular membranes (12, 29). Cells were disrupted by Dounce homogenization, and fractions were obtained by a series of centrifugation steps. The polypeptides contained in the various fractions are shown in Fig. 4A. Unbroken cells, nuclei, and large membrane fragments were pelleted (1,000 × *g* for 15 min; lane 1), and the smaller membranes were pelleted by centrifugation (45,000 × *g* for 1 h). The supernatant was then centrifuged (200,000 × *g* for 1 h), and the polypeptides in the pellet and "soluble" proteins in the supernatant are shown in Fig. 4A (lanes 2 and 3, respectively). Large membranes associated with the 1,000 × *g* pellet were separated from nuclei in a discontinuous sucrose gradient (55,000 × *g*, 1 h) and collected at a 0/30% interface (lane 4) and a 30/60% interface (lane 5). Membranes from the 45,000 × *g* pellet were fractionated on a discontinuous 20 to 60% sucrose gradient (95,000 × *g*, 14 h), and in Fig. 4A (lanes 6 to 10) are shown the polypeptides associated with these membrane fractions in increasing order of membrane density. It has previously been shown that the membranes found in Fig. 4A (lanes 7 and 8) are largely derived from the plasma membrane and smooth endoplasmic reticulum (12). Regardless of the precise origin of each fraction in this separation procedure, it can be seen that NB is associated with the same membrane fractions as are HA and NA.

To determine whether NB has properties of an integral membrane protein, the fraction shown in Fig. 4A (lane 5) was treated with various concentrations of Triton X-100 and KCl and separated by centrifugation (150,000 × *g* for 30 min) into a pellet and supernatant. Both 2% Triton X-100 and 0.5 M KCl were required to solubilize NB (Fig. 4B, lanes E). It has been shown previously that detergent plus high salt is needed to solubilize both viral and cellular membrane proteins, e.g., HN and F of paramyxoviruses and HA, NA, and M_2 of influenza A viruses (14, 29, 46).

NB is expressed at the cell surface with an extracellular NH_2 terminus and cytoplasmic COOH terminus. The data described above suggest that NB is an integral membrane glycoprotein anchored in membranes by the single hydrophobic region located near the NH_2 terminus. To examine whether NB is expressed at the infected-cell surface and to determine the topography of NB in membranes, intact cells

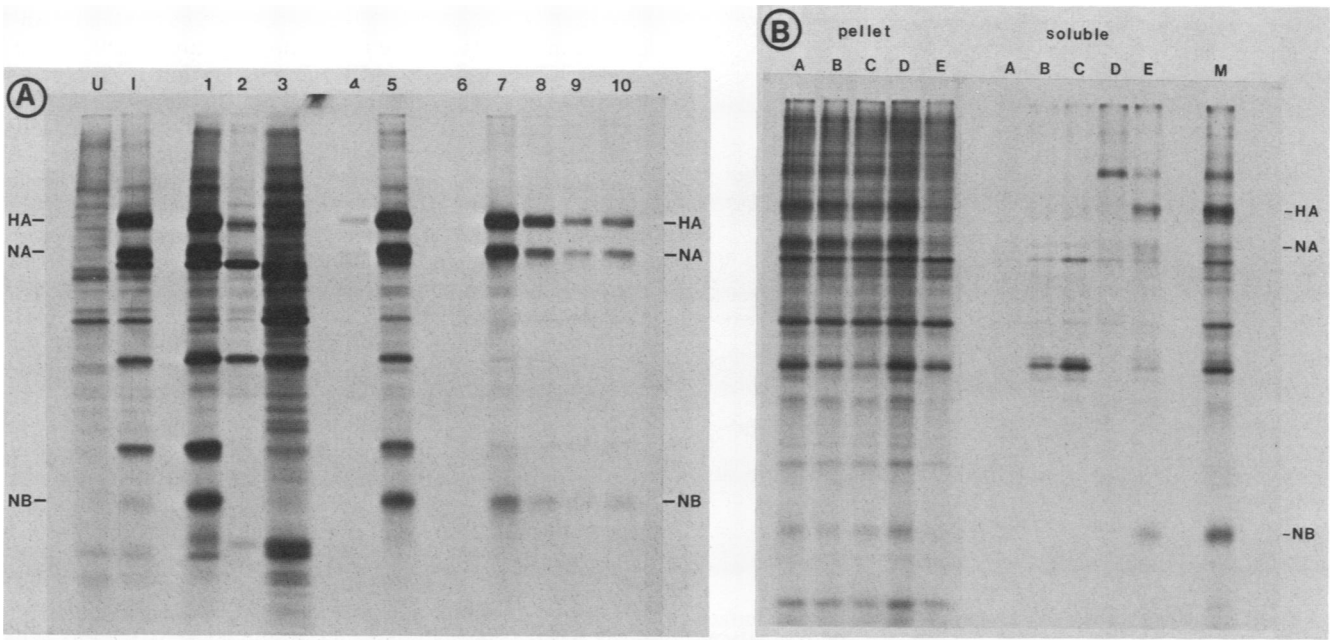

FIG. 4. (A) NB is found in the same membrane fractions as HA and NA. CV1 cells were infected with influenza B/Lee/40 and labeled with [^{35}S]cysteine from 8 to 10 h p.i. Cells were lysed and fractionated by the method of Hay (12). Lanes: U, mock-infected-cell lysate; I, total infected-cell lysate. Polypeptides found in various cell fractions are as follows. Lanes: 1, nuclear pellet (1,000 × *g* for 15 min); 2, 200,000 × *g* 1 h, pellet; 3, 200,000 × *g*, 1 h, supernatant; 4 and 5, large membranes from a 1,000 × *g*, 15-min pellet were centrifuged at 55,000 × *g* for 1 h and separated on a 30 to 60% sucrose gradient; 4, 0 to 30% sucrose interface; 5, 30 to 60% sucrose interface; 6 to 10, membranes collected from the 1,000 × *g*, 25-min supernatant were pelleted at 45,000 × *g* for 15 min and separated on a 20-25-30-35-40-60% sucrose step gradient at 95,000 × *g* for 14 h. Polypeptides from each fraction collected are shown in order of increasing membrane density from the top of the gradient (lane 6) to the bottom (lane 10). (B) NB is an integral membrane protein. Membranes from lane 5 in panel A were adjusted to the conditions indicated below, incubated at room temperature for 20 min, and then centrifuged at 150,000 × *g* for 30 min. The pellets were solubilized in electrophoresis sample buffer. The supernatants were made 10% trichloroacetic acid, and the proteins were precipitated. The trichloroacetic acid pellets were solubilized in electrophoresis sample buffer, and the pH was corrected to neutral. Lanes: A, no treatment; B, 0.5 M KCl; C, 1.0 M KCl; D, 2% Triton X-100; E, 0.5 M KCl–2% Triton X-100; M, TCA precipitated, untreated membrane fraction, not subjected to centrifugation.

were treated with proteinase K. [^{35}S]cysteine-labeled infected MDCK cells were gently scraped from tissue culture plates and incubated with 100 μg of proteinase K per ml for 40 min at 37°C. It was found that most of the HA and NA were selectively removed as would be expected for surface spike glycoproteins (Fig. 5, compare lanes 1 and 2) and that viral proteins known to reside inside the cell (e.g., NP, NS_1, and M) were largely protected from protease digestion. A loss in the amount of NB was observed with the appearance of several new bands (Fig. 5, compare lanes 1 and 2). To determine whether any of the new bands were related to NB, the samples were immunoprecipitated with the site-specific COOH-terminal CNB antisera. After proteinase K treatment of the cell surface, some NB was found in a "trimmed" form (Fig. 5, lane 6 [band d] of M_r ~10,000. The large shift in mobility from NB (band a) to trimmed NB (band d) would be expected if proteinase K removed a peptide containing carbohydrate residues. These data suggest that some of the NB molecules are accessible to digestion with proteinase K at the cell surface and, because of the specificity of the CNB antisera, make it likely that the NH_2 terminus of NB, containing the sugar chains, is exposed at the cell surface. To attempt to obtain a complete conversion of NB to the trimmed form, higher concentrations of protease and longer incubation times were used, but these resulted in unacceptable levels of cell lysis.

As an alternative approach to show that NB is expressed at the cell surface, intact cells were treated with endo F to determine the loss of carbohydrate from NB. As shown in the direct lysate (Fig. 5, lane 3) and in lysates immunoprecipitated with CNB antisera (Fig. 5, lane 7), two new bands were observed: a minor component band b (M_r, ~15,000) and a major component band c (M_r, ~11,500). Band c has identical mobility to that observed for NB immunoprecipitated from infected-cell lysates and treated with endo F (Fig. 3). Band b is thought to result from incomplete digestion with the enzyme and probably represents a form of NB that has lost one carbohydrate chain (see Fig. 8). The reproducible large increase in the amount of radioactivity in NB band c after endo F treatment of the cell surface was unexpected, but in influenza B virus-infected MDCK cells we have consistently observed a diffuse region of bands (M_r, ~35,000 to ~50,000) (Fig. 5, lanes 5 and 6) after precipitation with CNB or DNB antisera. These bands were lost during endo F treatment of the cell surface, and concomitantly a large increase in the amount of band c was observed (Fig. 5, lane 7). The CNB antisera did not precipitate any polypeptides from uninfected cells treated with endo F (data not shown), thus eliminating the unlikely possibility that endo F treatment of cells reveals an antigenic epitope of a host polypeptide that is recognized by the site-specific CNB antisera and that also has the same mobility as endo F-treated NB. Thus, it seems likely that NB interacts with itself or other molecules through the MDCK cell-specific carbohydrate moieties.

To further examine the NH_2-terminal region of NB ex-

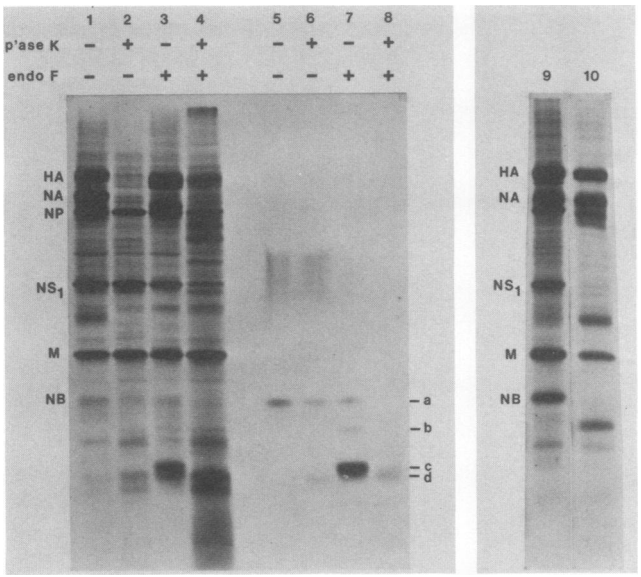

FIG. 5. Evidence for the expression of NB on the cell surface with its NH_2 terminus and carbohydrate chains exposed. MDCK cells were infected with influenza B/Lee/40, labeled with [^{35}S]cysteine, scraped from plates, and resuspended in phosphate-buffered saline. The intact cells were untreated (−) or treated (+) at 37°C with enzymes indicated above the lanes as follows: p'ase K, 100 μg of proteinase K per ml for 40 min; endo F, 1 U endo F for 3 h. Lanes 1 to 4 are direct lysates of the cells. Lanes 5 to 8 show the immunoprecipitation with CNB peptide antibody of the samples shown in lanes 1 to 4. Protein bands immunoprecipitated by CNB antibody are noted by lower case letters (a to d) and are discussed in the text. The amounts of lysates used were adjusted to make the level of NS protein approximately equivalent to correct for cells lost in the washing procedure, except for the immunoprecipitation in lane 8 where the available cells were used. Lanes 9 and 10 show results with a crude preparation of microsomes prepared from infected cells and treated with trypsin as described in Materials and Methods. Lane 9, No trypsin treatment; Lane 10, treatment with 100 μg of trypsin per ml.

posed at the cell surface, we attempted to treat intact cells sequentially with endo F and proteinase K. These treatments make the cells very fragile, and it is difficult to avoid considerable losses of cells during the procedure. Nonetheless, by selecting endo F and proteinase K conditions we were able to detect some remaining NB-specific bands c and d (Fig. 5, lanes 4 and 8).

To obtain indirect evidence that the 60 amino acids on the COOH-terminal side of the hydrophobic domain are exposed in the cytoplasm, a crude preparation of microsomes from [^{35}S]cysteine-labeled infected cells was isolated, treated with trypsin, and pelleted through a sucrose gradient. Our previous experiments have shown that greater than 90% of microsomal vesicles prepared in this manner are oriented outside-in as compared to the orientation of the cell surface (56). Trypsin treatment caused an expected slight increase in mobility in HA and NA due to digestion of their cytoplasmic tails, and a complete loss of cytoplasmic NS_1 was found associated with the crude microsome preparation (Fig. 5, lane 10). A small amount of cytoplasmic M protein was not digested by trypsin. However, trypsin treatment of microsomes caused a complete loss of NB compared to untreated microsomes (Fig. 5, lane 9). To examine whether any new band related to NB could be detected, the trypsin-treated microsomes were immunoprecipitated with the site-specific CNB antisera and DNB antisera but no protected band was observed (data not shown). This suggests that the epitopes recognized by the CNB antibody and the DNB antibody had been removed by trypsin digestion and is consistent with 60 amino acids residing on the cytoplasmic side of the membrane. The absence of a protected fragment containing the 18 NH_2-terminal residues and the membrane-spanning domain by the DNB antibody suggests that this region of NB is a poor antigenic epitope in gel-purified NB.

Expression of NB and NA from an SV40 recombinant virus. To further examine the synthesis and transport of NB to the plasma membrane and to provide a means of examining the phenotype of genetically engineered mutants in NB, the cDNA to influenza B virus RNA segment 6 was expressed in eukaryotic cells by using an SV40 late-region replacement vector. In influenza B virus-infected cells, both NB and NA are translated from a bicistronic mRNA derived from RNA segment 6 (48). Therefore, it was expected that both NB and NA would be expressed when the cDNA copy of RNA segment 6 was transcribed under the control of the SV40 late-region promoter and polyadenylation signal. The vector used, SV93 (see Materials and Methods), is very similar to SV103, described previously (38), except for restriction endonuclease sites available for cloning insert cDNAs. The G/C tails were removed from the 5′ end (mRNA sense) of the cDNA of RNA segment 6 (pBNA1 [49]) by controlled nuclease *Bal* 31 and restriction endonuclease digestions. Two deletions were used in the experiments described here. pSV93BNAΔ4 contains a deletion of three influenza virus-specific nucleotides, and pSV93BNAΔ39 contains a deletion of 38 influenza virus-specific nucleotides, both from the 5′ end of the DNA in the mRNA sense.

The recombinant SV40-B/NB/NA DNA molecules with the B/NB/NA 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 (SV*dl*1055) (40), and lytic stocks of virus were prepared.

When the SV40-B/NB/NA (mRNA and vRNA sense)-infected CV1 cells were labeled with [^{35}S]methionine at 48 h p.i. for 3 h and immunoprecipitated with NA antiserum (Fig. 6, left 5 lanes), a band of identical mobility to that of NA, precipitated from B/Lee/40 virus-infected cells, was detected from the mRNA sense vectors (Δ4 and Δ39) but not from the vRNA sense vector or from uninfected cells. To examine for the synthesis of NB, the vector-infected CV1 cells were labeled at 48 h p.i. for 3 h with [^{35}S]cysteine and immunoprecipitated with DNB antiserum. A band of identical mobility to NB from influenza virus-infected cells was detected with the mRNA sense vectors (Δ4 and Δ39) but not from the vRNA sense vector or from uninfected cells, indicating that NB was expressed from the vector and glycosylated normally (Fig. 6, right 5 lanes). It has been reported that NB has a short half-life (<1 h) in influenza B/Lee/40-infected HeLa cells (47), but in influenza B virus-infected CV1 and MDCK cells NB has a half-life of 4 to 5 h (data not shown) and NB does not turn over appreciably during the 3-h labeling period used in these SV40 recombinant virus experiments. In influenza B virus-infected cells, NB and NA are synthesized in approximately equimolar amounts (48). The relative amounts of NA and NB shown in Fig. 6 do not directly reflect amounts of NA and NB synthesized by the vector, since different radioactive isotopes and antisera of different titers were used.

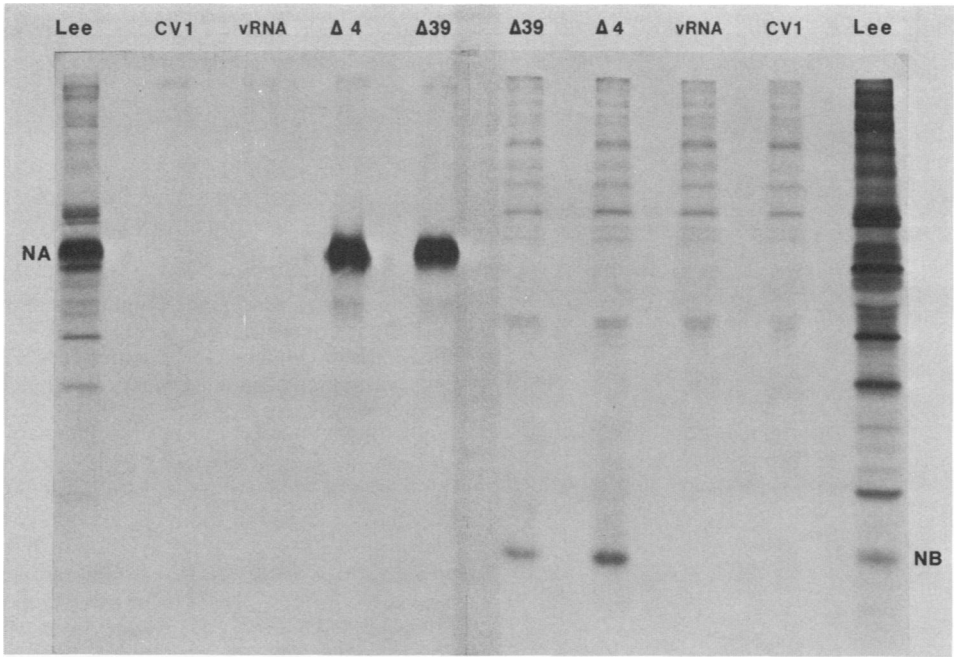

FIG. 6. Expression of NA and NB proteins from an SV40 vector. CV1 cells were infected with the SV40 recombinant viruses, labeled with [^{35}S]methionine (left five lanes) or [^{35}S]cysteine (right five lanes) and immunoprecipitated. The five lanes on the left side of the figure were immunoprecipitated with NA antibody. The right-side lanes (with the exception of the last lane) were immunoprecipitated with DNB antibody. Right side lane Lee is a direct lysate. Lanes: Lee, cells infected with B/Lee/40; CV1, mock-infected CV1 cells; vRNA, SV93BNAΔ4 vector with B/NB/NA DNA insert in the missense orientation with respect to SV40 late-region transcription; Δ4, SV93BNAΔ4 recombinant virus; Δ39, SV93BNAΔ39 recombinant virus. Lanes Δ4 and Δ39 contain the B/NB/NA DNA in the mRNA sense with respect to SV40 late-region transcription.

Identification of the precise carbohydrate sites in NB by using site-specific mutagenesis and expression in eucaryotic cells. The predicted amino acid sequence of NB (Fig. 1) indicates that there are four potential sites (Asn-X-Ser/Thr) for the addition of N-linked carbohydrate. The biochemical data described above suggest that NB is expressed at the cell surface and oriented in membranes with ~18 NH_2-terminal residues exposed and that this region of NB is glycosylated. To obtain direct information on the carbohydrate addition sites used and to confirm by a genetic means the orientation in membranes, the Asn residue in each of the four Asn-X-Ser/Thr sequences was changed to a Ser residue by oligonucleotide-directed site-specific mutagenesis of a specific fragment of pSV93BNAΔ4 in bacteriophage M13mp19. The mutants were as follows: (i) CHO1, residue 3, Asn changed to Ser; (ii) CHO2, residue 7, Asn changed to Ser; (iii) CHO3, residue 48, Asn changed to Ser; (iv) CHO4, residue 93, Asn changed to Ser; and (v) CHO1,2, a double mutant in which the Asn at residues 3 and 7 were both changed to Ser. The nucleotide sequences and predicted amino acid sequences of the wild-type and mutant strains are shown in Fig. 7. The mutants, once constructed into the SV40 vector described above, were expressed in CV1 cells. The identification of the mutants was expected to be unambiguous due to the large difference in mobility between NB (M_r, ~18,000) and endo-F-treated NB (M_r, ~11,500) (Fig. 5). The phenotype of the mutants that had lost a glycosylation site was predicted to be an NB-specific polypeptide of intermediate gel electrophoretic mobility (i.e., M_r, ~14,000 to 15,000).

SV40-recombinant virus-infected CV1 cells were labeled with [^{35}S]cysteine and immunoprecipitated with CNB antibody. NB (M_r, ~18,000) expressed from the wild-type pSV93BNAΔ4 vector is indicated by an arrow in Fig. 8A, lane A. NB expressed from the wild-type vector was treated with endo F and immunoprecipitated from cell lysates. The mobility of NB was shifted to an M_r of ~11,500 (Fig. 8A, lane B) as observed from endo F treatment of influenza virus-infected cells (Fig. 5). The mutants CHO1 and CHO2 expressed major new NB-specific bands of ~14,000 to 15,000 M_r (lanes 1 and 2, respectively), whereas mutants CHO3 and CHO4 expressed major NB-specific bands of ~18,000 M_r (lanes 3 and 4, respectively. The double mutant CHO1,2 expressed a major NB-specific ~10,500-M_r band. Thus, these data would indicate that the NH_2-terminal Asn residues 3 and 7, which are exposed at the cell surface, are both used for the addition of carbohydrate. It can be seen that the mutants CHO1 and CHO2 express additional NB-related species not seen with the wild-type vector or else seen in much less abundance (Fig. 8A, lanes 1 and 2). One of the additional bands migrates with a mobility which is similar but not identical to that of glycosylated wild-type NB. To rule out the trivial explanation that these species had arisen because of contamination of the mutants, the nucleotide sequence of NB in the vector DNA molecules was obtained immediately before transfection into the cells. Additionally, the mutation in CHO1 eliminates the AUG codon for NA (Fig. 7). No expression of NA was detected with CHO1 at a level that would have detected 1% wild-type expression. The observation that these additional bands are lacking in mutant CHO1,2 (no carbohydrate chains) suggests that they arise by heterogeneity in the composition of the carbohydrate chains, especially when there is only one sugar chain attached to NB, which leads to different electrophoretic forms on gel analysis. We examined further that the extra NB-specific

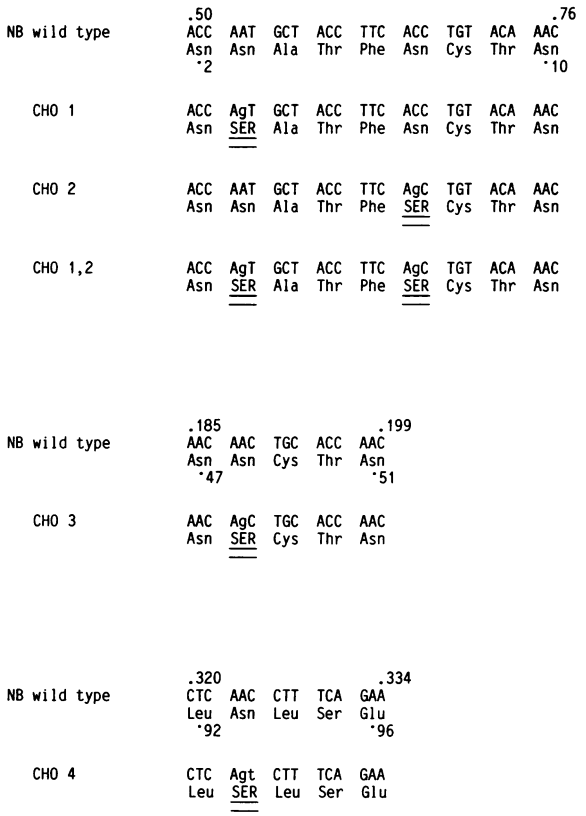

FIG. 7. NB glycosylation site mutations. The nucleotide sequences and deduced amino acid sequence are shown for the regions surrounding the four potential N-linked glycosylation sites of NB. The sites are numbered 1 to 4 with respect to their distance from the protein NH_2 terminus. The nucleotide positions in NB (from reference 49) are shown above the sequence, where the A residue of the NB AUG initiation codon is nucleotide 47. The amino acid residue numbers (48) are indicated below the amino acid sequence. The mutants (CHO) are designated after the glycosylation site(s) altered. The nucleotides changed by mutagenesis are shown as lower-case bases, and the changed amino acid (a serine residue in each case) is shown in upper-case letters and is double underscored.

bands and changes in the electrophoretic mobility of NB in CHO1, CHO2, and CHO1,2 were due to effects of the lack of one or two carbohydrate chains. The NB-specific proteins expressed by the vectors were treated with endo F and precipitated with CNB antibody. A single major species of NB (M_r ~11,500) was observed in all of the mutants, except for CHO2, in which the digestion with endo F is thought to be incomplete. One possibility for the heterogeneity of NB mobility observed for CHO1 and CHO2 (Fig. 8A) could be sulfation of the carbohydrate, a modification known to occur for HA and NA of influenza virus (36). The degree of sulfation may lead to different electrophoretic forms on SDS-polyacrylamide gels. A precedent for this type of phenomenon is found with many phosphoproteins in which multiple electrophoretic bands are found on gels and is dependent on the level of phosphorylation, e.g., M of Sendai virus (23) and NS of vesicular stomatitis virus (18). There was a small variation in the mobility of NB after endo F treatment between the mutants and the wild-type NB (Fig. 8B). This was not unexpected since in each mutant an Asn residue had been changed to a Ser, and it is well established, especially with influenza virus polypeptides (reviewed in references 21), that single-amino-acid changes can slightly alter the mobility of a polypeptide on gels.

DISCUSSION

The data presented here indicate that NB (100 amino acids), the third glycoprotein of influenza B virus in addition to HA and NA, is an integral membrane protein expressed at the infected-cell surface. A single hydrophobic domain (residues 19 to 40) anchors the protein in membranes and ~18 NH_2-terminal amino acids containing two endo-H-sensitive carbohydrate chains are exposed at the cell surface. A schematic diagram of the structure of NB is shown in Fig. 9. This was demonstrated by biochemical methods and the data complemented by a genetic approach involving deletion of potential glycosylation sites in the NB cDNA by using site-specific mutagenesis and expression of the altered proteins in cells. The gel mobility differences in the expressed polypeptides allowed the identification of the precise carbohydrate sites used for glycosylation of NB (Asn at residue 3 and Asn at residue 7). This technique should be generally applicable whenever a cDNA clone of a transmembrane protein is available and should be particularly useful in determining the glycosylated domains exposed extracellularly of proteins that span the membrane more than once. The well-characterized N-linked carbohydrate addition sites of the vesicular stomatitis virus G protein have also been deleted by using site-specific mutagenesis (31), but the purpose of these experiments was to determine the role of sugar chains on transport of protein G to the cell surface.

The role of NB in influenza B virus infections is not known. Although NB is readily detected in infected cells, it has not been detected in purified virions; this may be dependent on the sensitivity of the methods employed (48). The absence of NB in any great amount from virions as compared to HA and NA suggests that there must be an exclusion mechanism for NB before or during the budding process. It will be of great interest to determine the geographic distribution of NB on the cell surface with respect to budding virions. The cell surface localization of NB makes it unlikely that the protein is involved in transcription or replication of RNA. A role in organizing proteins at the cell surface to form patches of viral proteins or in the budding process would seem more likely.

Influenza A and B viruses both contain eight RNA segments, and at least nine polypeptides encoded by influenza B viruses have counterparts in influenza A virus: PB1, PB2, PA, HA, NA, NP, M_1, NS_1, and NS_2 (reviewed in references 1 and 21). A major difference between influenza A and B viruses is the finding of the NB glycoprotein in influenza B viruses. NB is translated from a bicistronic mRNA derived from RNA segment 6 that also encodes NA by using overlapping reading frames (28, 48). A second open reading frame has not been found on RNA segment 6 of influenza A viruses. However, influenza A viruses encode the M_2 polypeptide that is translated from a spliced mRNA derived from RNA segment 7 and overlaps the region encoding M_1 (24, 27). No counterpart for M_2 has been reported in influenza B virus-infected cells, although RNA segment 7 of influenza B virus, which encodes the M_1 protein, does contain a second overlapping reading frame (4). It has recently been shown that influenza A virus M_2 protein is an integral membrane protein expressed at the infected-cell surface (29, 56). The results presented here for NB of influenza B virus show that it has structural similarities with M_2 of influenza A virus. These include the following. (i) NB and M_2 are of similar

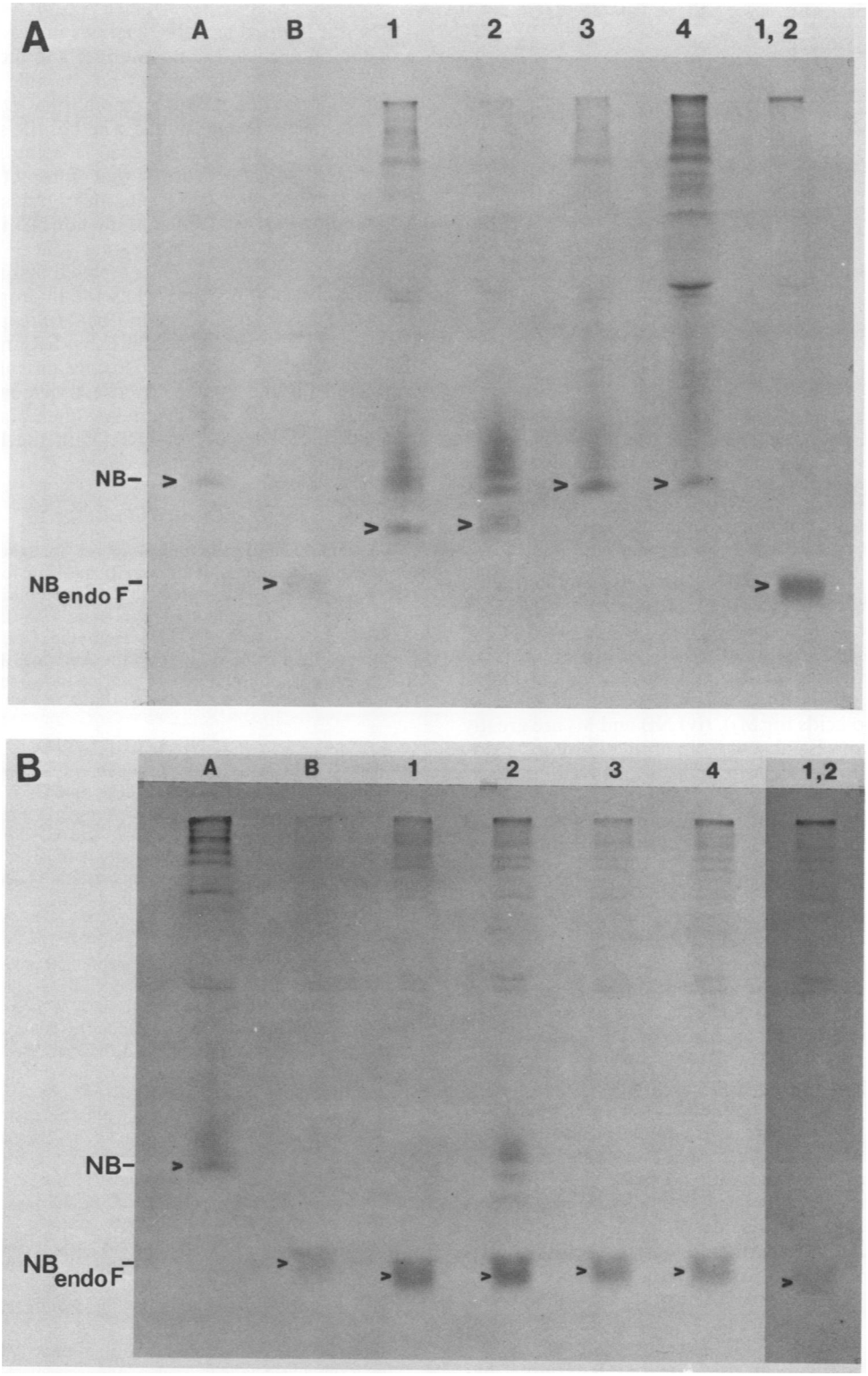

FIG. 8. (A) Expression of NB glycosylation mutants in an SV40 recombinant vector. CV1 cells were infected with the SV40 recombinant viruses containing the glycosylation site mutants in NB, labeled with [^{35}S]cysteine at 48 h p.i., lysed, and immunoprecipitated with CNB antibody. Lanes: A, wild-type NB expressed from SV93BNAΔ4; B, wild-type NB expressed from SV93BNAΔ4 and the cell lysate treated with 0.25 U of endo F before immunoprecipitation. Lanes: 1 to 4, expression of the mutant NB from the vectors containing the CHO1, CHO2, CHO3, and CHO4 mutations, respectively; 1,2, expression of NB from the double mutation CHO1,2. (B) Endo F treatment of NB expressed by the CHO mutants. CV1 cells were infected with the SV40 recombinant vectors, labeled with [^{35}S]cysteine, and lysed as described above. Aliquots of each lysate were treated with 0.25 U of endo F for 12 h at 37°C before immunoprecipitation. Lanes: A, wild-type untreated NB expressed from SV93BNAΔ4; B, wild-type NB expressed from SV93BNAΔ4; 1 to 4, expression of the mutant NB from the vectors containing CHO1, CHO2, CHO3, and CHO4 mutations, respectively; 1,2, expression of NB from the double mutation CHO1,2.

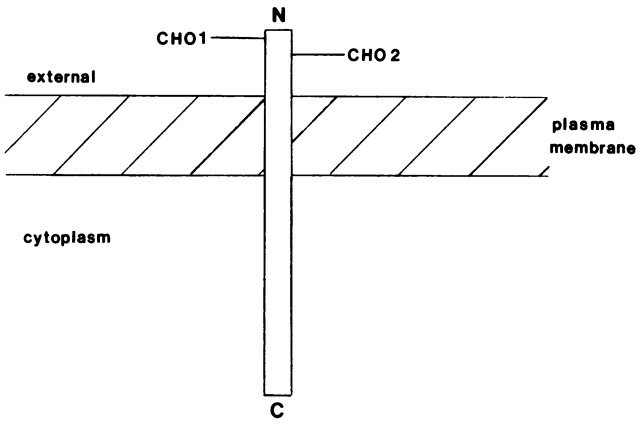

FIG. 9. Schematic diagram of NB indicating its orientation in membranes. The length of the amino acid backbone of the glycoprotein is drawn to scale, assuming the anchor domain spanning the membrane is the entire uncharged region. N and C denote the NH_2 and COOH termini and the glycosylation sites shown to be used are indicated at their relative positions from the NH_2 terminus.

sizes (100 and 97 amino acids, respectively) and contain a single internal hydrophobic domain. (ii) NB and M_2 are integral membrane proteins and localized on the infected-cell surface. (iii) NB and M_2 are oriented with a short NH_2-terminal region exposed at the cell surface and a longer COOH-terminal cytoplasmic domain (~60 amino acids for NB and ~53 amino acids for M_2). (iv) NB and M_2 are greatly underrepresented or else absent from purified virions. However, NB and M_2 do not share primary sequence homology, and NB is glycosylated whereas M_2 is not (56). Whether the similarity of structural features between NB and M_2 is fortuitous or whether this reflects a common function is not known.

Integral membrane proteins are found in several topographical forms with respect to the lipid bilayer. Many viral and cellular glycoproteins have a cleaved NH_2-terminal signal sequence, span the membrane once, and are oriented with a large NH_2-terminal extracellular domain and a small COOH-terminal cytoplasmic tail, e.g., influenza virus HA, paramyxovirus F, vesicular stomatitis virus G, Semliki Forest viruses E_1 and E_2 (10, 37, 41, 44). Some glycoproteins have a single hydrophobic domain (extended signal anchor) in an NH_2-terminal region, span the membrane once, and have a large COOH-terminal extracellular domain and a small NH_2-terminal cytoplasmic tail, e.g., influenza virus NA, paramyxovirus HN, and human asialoglycoprotein receptor (8, 15, 16, 51). Several cellular membrane proteins contain uncleaved signal sequences near the NH_2 terminus and span the membrane several times with cytoplasmic NH_2 and COOH termini, e.g., human glucose transport protein, erythrocyte band 3, MP26 of the bovine lens fiber membrane, Ca^{2+}/Mg^{2+}-ATPase, and the catalytic subunit of NA^+/K^+-ATPase (11, 19, 32, 35, 50). However, the known examples of proteins with an uncleaved NH_2-terminal hydrophobic domain that anchors the protein in membranes with an NH_2-terminal extracellular domain and COOH-terminal cytoplasmic domain is much smaller. Influenza B virus NB, influenza A virus M_2 (29), and, probably, avian erythroblastosis virus gp74v-erbB (13, 42, 55) each have a single NH_2-terminal-region hydrophobic domain that anchors the protein in the lipid bilayer. Bovine opsin does not contain a cleavable NH_2-terminal signal, spans the membrane several times, and has an extracellular NH_2 terminus. The opsin internal signal sequences interact with the signal recognition particle (SRP) to target and facilitate cotranslational insertion of the nascent polypeptide chain across the rough endoplasmic reticulum membrane (9, 39).

It remains to be determined whether the single, uncleaved hydrophobic domain of NB (and M_2) can be defined as an "extended signal anchor" that interacts with SRP to cotranslationally insert NB into membranes and then acts as a "stop transfer" sequence to anchor NB in membranes. If this is the case, it is interesting that ~78% of the NB polypeptide chain would be completed before the interaction with SRP occurs, because when the hydrophobic domain is exposed, a further ~40 amino acids are protected by the ribosome (53). It is also possible that SRP may interact with the hydrophobic domain posttranslationally as has been suggested for the human glucose transporter protein (35). Alternatively, NB may insert into membranes posttranslationally, independently of SRP, with the hydrophobic domain acting as an insertion sequence (2).

ACKNOWLEDGMENTS

We thank Diane Braun and Margaret Shaughnessy for excellent technical assistance. Christopher Richardson for synthesizing the NB oligopeptide, and Robert Webster for preparing antisera to NA. This research was supported by Public Health Service research awards AI-20201 and AI-23173 from the National Institutes of Health and by a grant-in-aid from the American Heart Association. R.A.L. is an established investigator of the American Heart Association.

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