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NH_2-terminal hydrophobic region of influenza virus neuraminidase provides the signal function in translocation

(sialidase/leader peptide/chimeric protein/membrane glycoprotein/hemagglutinin)

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ABSTRACT **Influenza virus neuraminidase (NA), unlike the majority of integral membrane proteins, does not contain a cleavable signal sequence. It contains an NH_2-terminal hydrophobic domain that functions as an anchor. We have investigated the signal function for translocation of this NH_2-terminal hydrophobic domain of NA by constructing chimeric cDNA clones in which the DNA coding for the first 40 NH_2-terminal hydrophobic amino acids of NA was joined to the DNA coding for the signal-minus hemagglutinin (HA) of influenza virus. The chimeric HA (N4OH) containing the NH_2 terminus of NA was expressed in CV1 cells by using a simian virus 40 late-expression vector. The chimeric HA is synthesized, translocated into the rough endoplasmic reticulum, and glycosylated, whereas HA lacking the signal sequence is present only in small amounts and is unglycosylated. These results clearly show that the NH_2 terminus of NA, in addition to its anchor function, also provides the signal function in translocation. However, the acquisition of complex oligosaccharides and the transport of N4OH to the cell surface are greatly retarded. To determine if the presence of two anchor sequences, one provided by NA at the NH_2 terminus and the other provided by HA at the COOH terminus of N4OH, was responsible for the slow transport, the NH_2 terminus of NA was fused to an "anchorless" HA. The resulting chimeric HA (N4OH482) contains the hydrophobic domain of NA at the NH_2 terminus but lacks the HA anchor at the COOH terminus. N4OH482 was synthesized and glycosylated; however, as with N4OH, the acquisition of complex oligosaccharides and the migration to the cell surface are greatly retarded. Immunofluorescence data also support that, compared to the native HA, only a small amount of chimeric HA proteins is transported to the cell surface. Thus, the hydrophobic NH_2 terminus of NA, although capable of providing the signal function in translocation across the rough endoplasmic reticulum, interferes with the transport of the chimeric HA to the cell surface.**

Influenza virus contains two integral membrane proteins on its envelope, hemagglutinin (HA) and neuraminidase (NA) (1, 2). These two proteins have been used extensively in studying eukaryotic membrane biogenesis (3–11). Both influenza HA and NA are among the best-characterized integral membrane proteins. Complete amino acid sequences of HA and NA of a number of strains of influenza A and B viruses have been determined (12–17). In addition, three-dimensional structures, antigenic epitopes, glycosylation points, cleavage sites of HA, receptor binding sites for sialic acid residues for both HA and NA, as well as organization into trimeric (HA) or tetrameric (NA) structures have been defined (18–21). Although both HA and NA are inserted into viral membranes, the structural features of these two integral membrane proteins are quite different. HA, for example, possesses the structure of a classical integral membrane protein that contains hydrophobic amino acid sequences both at the NH_2 terminus and the COOH terminus. The NH_2-terminal hydrophobic amino acid sequence provides the signal function in translocation and is subsequently removed from the mature protein by proteolytic cleavage. The COOH-terminal hydrophobic sequence, on the other hand, serves to anchor the protein to the membrane. NA also possesses two stretches of hydrophobic sequence—one consisting of 29 amino acids (positions 7–35) at the NH_2 terminus (14, 16) and the other consisting of 16 amino acids (positions 420–435) in the proximity of the COOH terminus. However, unlike HA, the hydrophobic sequence at the NH_2 terminus is not cleaved from the mature protein. Furthermore, it has been shown that this NH_2-terminal sequence remains embedded in the membrane and serves as an anchor (22). It is not known, however, which hydrophobic sequence of NA provides the signal function in translocation across the rough endoplasmic reticulum (RER).

In this investigation, we have constructed chimeric cDNA clones in which the DNA coding for the NH_2-terminal hydrophobic sequence of NA of A/WSN/33 (H1N1) influenza virus is joined to the DNA coding for signal-minus HA as well as to the DNA coding for signal-minus and anchorless HA. Furthermore, the chimeric proteins encoded by these cDNA clones have been expressed in CV1 cells by using a simian virus 40 (SV40) late-expression vector (3), and the results show that the NH_2-terminal hydrophobic sequence of NA, in addition to its anchor function, also provides the signal function in translocation across the RER.

MATERIALS AND METHODS

Viruses, Plasmids, and Cells. Virus stocks of the A/WSN/33 strains of influenza virus were prepared in Madin–Darby bovine kidney (MDBK) cells as reported (3). Construction of cDNA clones containing the entire coding sequence of HA and NA as well as expression of HA (pHAX) and NA (pSNC) by using SV40 late-replacement vectors have been reported (3, 6). SVSal.32, which is defective in T-antigen expression (3), was used as a helper virus to complement the SV40 late-replacement vector in a lytic infection. CV1 and CV1P cells were cultured and infected with SV40 virus stocks as reported (3, 7).

Transfection and Preparation of Virus Stocks. CV1P cells (23) were transfected simultaneously with SV40 recombinant DNA and SVSal.32 DNA as described (3, 7). After complete lysis of cells (usually 12–14 days after infection), lysates containing both viruses were prepared. The lysate was passaged once to obtain the virus stock. All experiments were done in CV1 cells.

Intracytoplasmic and Cell Surface Immunofluorescence. At 48–72 hr after infection, cells infected with both recombinant

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Abbreviations: NA, neuraminidase; HA, hemagglutinin; RER, rough endoplasmic reticulum; SV40, simian virus 40.

and helper viruses were assayed by indirect immunofluorescence. For intracellular staining, cells were fixed in acetone/methanol, 1:1 (vol/vol), and for cell surface staining, cells were fixed with 3% (wt/vol) paraformaldehyde (3, 7). Fixed cells were stained with either anti-WSN antibodies made in rabbit or anti-HA monoclonal antibodies. Cells were subsequently stained with fluorescein-conjugated anti-rabbit or anti-mouse immunoglobulins (3, 6, 7).

Radiolabeling and Analysis of Polypeptides in Polyacrylamide Gels. At 40–45 hr after infection, cells were labeled with L-[^{35}S]methionine at 50 μCi/ml (1 Ci = 37 GBq). For tunicamycin treatment, cells were first treated with tunicamycin (2 μg/ml) for 1 hr at 37°C and then labeled with L-[^{35}S]methionine in the presence of tunicamycin. Labeled cells were scraped from dishes and lysed with RIPA buffer (3, 7) for 10 min at 0°C. Nuclei were removed and the superantant was incubated with anti-WSN antibodies for 2 hr at 4°C. Antigen–antibody complexes were isolated by using staphylococcal protein A-Sepharose (3, 7). Cell supernatants were collected after labeling and centrifuged two times for 15 min at 4°C in an Eppendorf Microfuge, 5× concentrated RIPA buffer was added, and the cell supernatants were immunoprecipitated as above.

Treatment with Endoglycosidase H. Immunoprecipitated samples were treated with endoglycosidase H as described (7).

RESULTS AND DISCUSSION

Construction of Chimeric DNA Clones. To test the signal property of the NH_2-terminal hydrophobic sequence of NA in translocation, the following constructions were made (Fig. 1): cDNA coding for signal-minus HA (pHT548) was constructed by deleting the entire untranslated region as well as the entire signal sequence of HA and adding the codon for methionine (ATG) just before the amino acid sequence of mature HA. The construction of pHT548 has been described (24). The signal-minus HA DNA was inserted into the late region of SV40 either in the same reading frame as the first 9 amino acids of the agnoprotein and the *Eco*RI linker (pSHA548, Fig. 1*C*) or in a different reading frame in which the ATG codon of HA548 would serve as the initiation of translation (pSH548, Fig. 1*D*). Specifically, the HA insert of pHT548 (24) obtained by partial *Eco*RI treatment was ligated into the *Eco*RI site of either pA11SVL3 (3) or pA11SVL4. pA11SVL4 differs from pA11SVL3 in the *Eco*RI linker sequence by one extra nucleotide (cytosine) at the 5′ end. Finally, in the chimeric construction (pSN4OH, Fig. 1*A*), the DNA encoding the untranslated region as well as the entire NH_2-terminal hydrophobic sequence of NA (positions 1–40) were joined in frame to the signal-minus HA DNA and inserted into the late-replacement SV40 vector. The sequences at the junction sites were verified. Predicted amino acid sequences at the NH_2 terminus are shown in Fig. 1 (*Inset*). Therefore, except for pHAX, which contains the native HA insert, 17 amino acids at the NH_2 terminus of HA were replaced either by a methionine residue only (pSH548), by 9 amino acids from the SV40 agno leader and *Eco*RI linker sequences plus a methionine (pSHA548), or by 40 amino acids of the NH_2 terminus of NA and a methionine (pSN4OH). The 40 amino acids at the NH_2 terminus of NA consist of 6 amino acids from the cytoplasmic domain, 29 hydrophobic

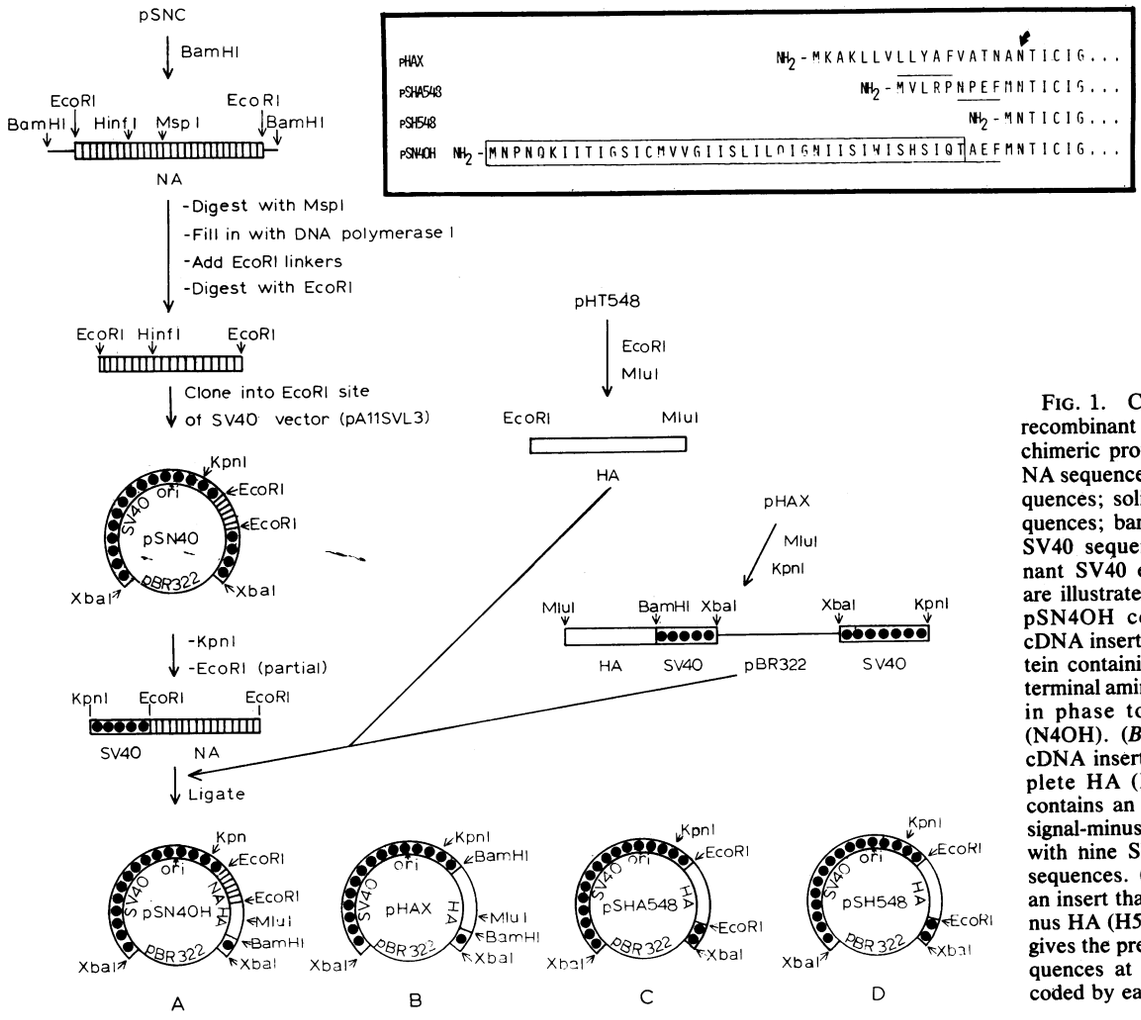

FIG. 1. Construction of SV40 recombinant DNAs that express chimeric proteins. Hatched bars, NA sequences; open bars, HA sequences; solid lines, pBR322 sequences; bars with solid circles, SV40 sequences. The recombinant SV40 expression plasmids are illustrated at the bottom. (*A*) pSN4OH contains a chimeric cDNA insert that codes for a protein containing the first 40 NH_2-terminal amino acids of NA linked in phase to signal-minus HA (N4OH). (*B*) pHAX contains a cDNA insert that codes for complete HA (HA). (*C*) pSHA548 contains an insert that codes for signal-minus HA (H548) in phase with nine SV40 agno and linker sequences. (*D*) pSH548 contains an insert that codes for signal-minus HA (H548). The boxed insert gives the predicted amino acid sequences at the NH_2 termini encoded by each plasmid.

amino acids of the transmembrane domain, and 5 amino acids after the transmembrane domain (12).

Expression of Chimeric HA in CV1 Cells. Immunofluorescence studies using anti-HA monoclonal antibodies showed that chimeric HA (N4OH, Fig. 2*B*) containing the NH_2-terminal hydrophobic sequence of NA is expressed essentially to the same extent as native HA (Fig. 2*A*). However, the expression of the signal-minus HA alone (H548) or signal-minus HA containing the SV40 agno leader (HA548) could not be detected by immunofluorescence (data not shown), suggesting that little, if any, signal-minus HA is present in the cells. Essentially similar results were obtained by gel analysis of ^{35}S-labeled proteins after immunoprecipitation using either polyclonal or monoclonal anti-HA antibodies (Fig. 3). N4OH (Fig. 3, lane 6) has essentially the same mobility (M_r 70,000) as that of the native HA by polyacrylamide gel electrophoresis (Fig. 3, lane 3). On the other hand, both signal-minus HAs, H548 and HA548, are present in a much reduced amount (Fig. 3, lanes 1 and 2, arrow) and are smaller (M_r 62,000) in size.

Poor synthesis of the signal-minus HA polypeptide with or without the SV40 agno sequence is not likely to be due to a translational or transcriptional block caused by the presence of the agno and *Eco*RI linker sequences because both HA and NA can be expressed either directly, using the HA or NA ATG codon, or in phase with the SV40 agno and linker sequences using the agno ATG codon (3, 6). Moreover, SH548, unlike SHA548, was not in phase with the SV40 agno and linker sequences and, therefore, was expected to use its own ATG codon for initiating protein synthesis. However, the expression of both H548 and HA548 was very low. Furthermore, the amount of HA-specific mRNA in CV1 cells, as detected by hybridization, is essentially the same for all three constructions as well as for wild-type HA (data not shown). This suggests that a transcriptional block was not a major factor in accounting for the reduced expression of signal-minus HA. A greatly reduced expression of signal-minus HA has also been reported by others (5). We have also obtained a similar reduction in expression with a NA cDNA clone (SN26) that lacks the DNA encoding 26 amino acids at the NH_2-terminal end (3), suggesting that integral membrane proteins in general may require the NH_2-terminal portion of the signal sequence for proper expression and stability.

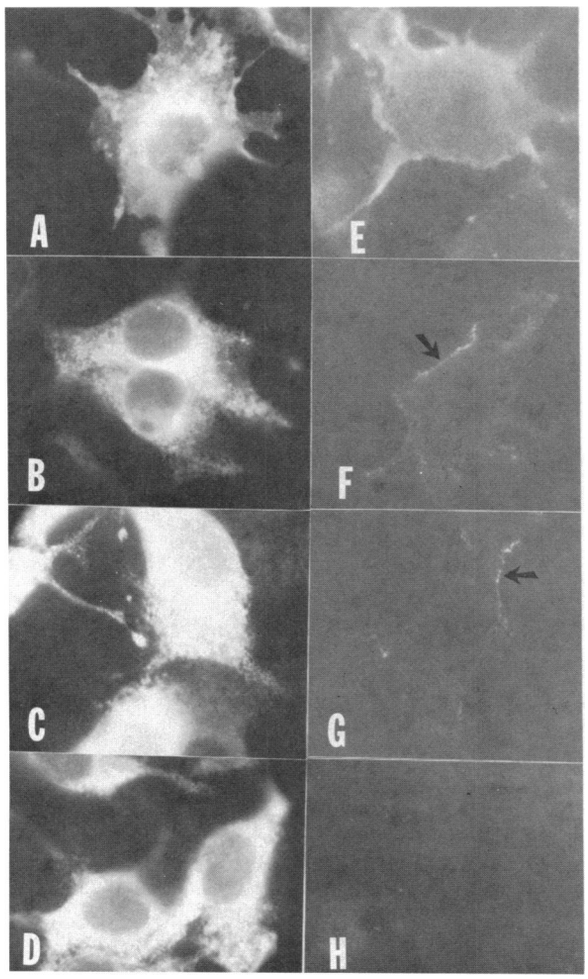

FIG. 2. Immunofluorescent antibody staining of CV1 cells infected with SV40 recombinant virus. Cytoplasmic staining of HA (*A*), N4OH (*B*), N4OH482 (*C*), and HA482 (*D*). Surface staining of HA (*E*), N4OH (*F*), N4OH482 (*G*), and HA482 (*H*). Arrows point to positive surface fluorescence in N4OH and N4OH482. (×210.)

Localization and Glycosylation of Chimeric HA. The main function of a signal sequence is to translocate the polypeptide across the membrane of the RER (25). Since these integral membrane proteins are cotranslationally glycosylated, glycosylation can be used to assay translocation across the RER. We therefore studied the translocation function of the NH_2-terminal NA sequence by determining the glycosylation of chimeric HA and wild-type HA in the presence and absence of tunicamycin. The results show that both the native HA and chimeric HA are glycosylated in the absence of tunicamycin (Fig. 3, lanes 3 and 6). These results clearly indicate that the NH_2-terminal NA sequence provides the function required for translocating the chimeric protein into the RER and, therefore, satisfies the criteria for a signal sequence. The signal-minus HA, on the other hand, made in the absence of tunicamycin (Fig. 3, lanes 1 and 2, arrow) has an M_r of 62,000, which is the same as that of the native HA made in the presence of tunicamycin (Fig. 3, lane 4). These data indicate that the signal-minus HA is not glycosylated and, therefore, is unlikely to be translocated across the RER. Data not reported here indicate that the large molecular weight protein (Fig. 3, lanes 1 and 2) that migrates in the same position as the glycosylated HA is not HA but rather an unglycosylated host protein contaminant.

To further determine if chimeric HA, like wild-type HA,

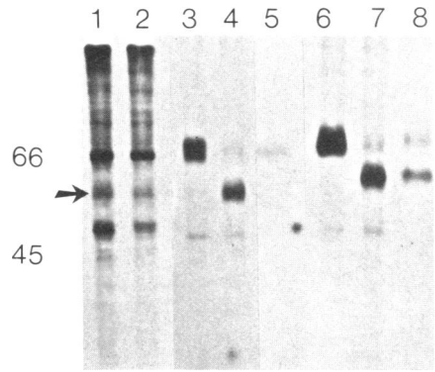

FIG. 3. Gel electrophoresis of native or chimeric HA proteins produced in CV1 cells infected with SV40 recombinant virus. CV1 cells were labeled with L-[^{35}S]methionine either in the presence (lanes 4 and 7) or absence (lanes 1, 2, 3, 5, 6, and 8) of tunicamycin for either 6 hr (lanes 1 and 2) or 2 hr, followed by a 4-hr chase (lanes 3–8). Lysates were immunoprecipitated with anti-WSN antibodies. Lane 1, H548; lane 2, HA548; lane 3, HA; lane 4, HA and tunicamycin; lane 5, HA and endoglycosidase H; lane 6, N4OH; lane 7, N4OH and tunicamycin; lane 8, N4OH and endoglycosidase H. Lanes 1 and 2 were run on a different gel than the others. Note that the native HA after endoglycosidase H treatment has the same molecular weight as that of HA without endoglycosidase H treatment (lanes 3 and 5), whereas the majority of N4OH has a reduced molecular weight after the endoglycosidase H treatment (lanes 6 and 8).

data indicate that the transport of the chimeric HA from the RER to the plasma membrane via the Golgi apparatus was greatly retarded and did not occur in most cells. Heterogeneity in transport of altered proteins to the cell surface has been previously reported for cells expressing altered vesicular stomatitis virus G proteins (26). Furthermore, as in cells expressing altered vesicular stomatitis virus G proteins, neither the percent of cells showing cell surface fluorescence nor the amount of cell surface fluorescence was different when the cells were examined at 48 or 72 hr after infection (data not shown).

Since HA acquires complex oligosaccharides that are resistant to endoglycosidase H after its transport to the Golgi apparatus, we wanted to determine if the chimeric HA was blocked prior to the acquisition of complex oligosaccharides. Digestion with endoglycosidase H indicated that the chimeric HA (Fig. 3, lane 8), unlike native HA (Fig. 3, lane 5), was predominantly of the high mannose type and only a small fraction contained complex sugars. Therefore, the transport of chimeric HA is blocked at some stage prior to the acquisition of complex oligosaccharides—i.e., either between the RER and the Golgi apparatus or between the *cis* and *trans* regions of the Golgi apparatus. The small fraction of endoglycosidase H-resistant N4OH protein (Fig. 3, lane 8) is probably related to the small fraction of chimeric protein found on the cell surface.

Cleavage of the NH_2 Terminus. Since the NH_2-terminal hydrophobic sequence of NA is not cleaved in mature NA, whereas that of HA is cleaved, it was of interest to see if the NA sequence in chimeric HA was processed. Gel analysis of unglycosylated chimeric HA and wild-type HA shows that the chimeric HA is larger than wild-type (Fig. 3, lanes 4 and 7), suggesting that the NA portion of chimeric HA is not cleaved; however, in the absence of protein sequence data, we cannot rule out processing completely. This suggests that cleavage of the signal sequence may not depend on the primary structure of the protein to which it is attached, even though the NH_2 terminus of mature HA (Asp-Thr-Ile . . .) is present in the chimeric protein. More likely, it is a property of the signal sequence itself and/or the junction sequence between the signal and the rest of the protein. Although the cleavage site of an individual protein is quite specific, both the signal sequence and cleavage site, among different proteins, vary extensively. Further experiments to analyze specificity of cleavage by site-specific mutation or by making specific chimeric signals are necessary.

The Role of One Anchor vs. Two Anchors in the Transport of Chimeric HA. Since the NA signal is not cleaved, the chimeric HA may remain attached at both ends. Thus, the new NH_2-terminal signal may disrupt the secondary or tertiary structure that may be important in the transport of HA from the RER. Because of the loop-like topology of HA with both NH_2 and COOH termini in close proximity to each other, it was postulated that the structure of HA was folded with both NH_2 and COOH termini attached to the membrane and that the cleavage of the HA signal occurred after formation of the HA structure (18). However, unlike native HA, both of the hydrophobic sequences in chimeric HA are likely to remain attached to the RER and, therefore, may cause improper folding of HA into its tertiary structure, which may interfere in transport. Furthermore, the chimeric HA will possess two cytoplasmic domains, which may also interfere with transport (26).

To test this possibility we constructed a chimeric cDNA (SN4OH482) in which the same NH_2-terminal NA sequences were linked to anchorless HA (Fig. 4). This HA clone contained 482 amino acids of HA but was missing the last 66 amino acids of HA2, which included the cytoplasmic domain (12 amino acids), the entire anchor region (24 amino acids), and 30 other amino acids. A stop codon was placed after amino acid 482 such that only one extra amino acid (leucine) was encoded before termination. A corresponding cDNA

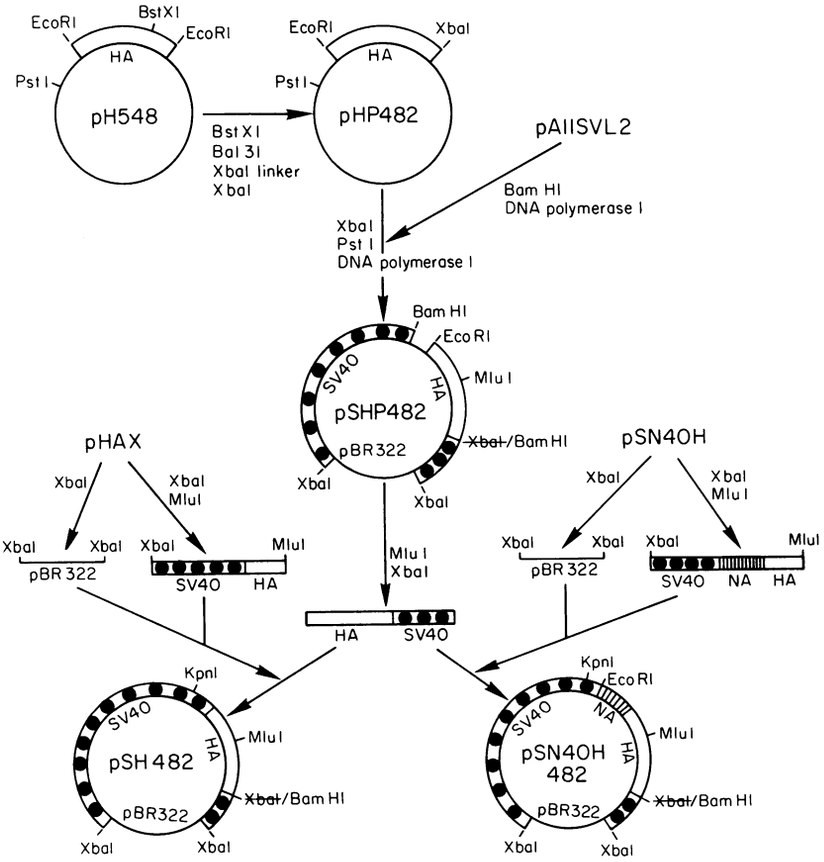

FIG. 4. Construction of SV40 recombinant DNAs that express anchorless HA and chimeric HA minus the COOH-terminal anchor. Signal-minus HA (pHA548) was treated with exonuclease BAL-31 to remove a portion of the COOH terminus of HA. An *Xba* I linker was added in phase with the DNA coding for HA to create a "stop codon." This truncated HA DNA was cloned into the late SV40 expression vector (pA11SVL2) and ligated to the 5′ ends of cDNA inserts in pHAX or pSN4OH to yield plasmids pSH482 and pSN4OH482, which code for proteins H482 and N4OH482, respectively. Symbols are as in Fig. 1.

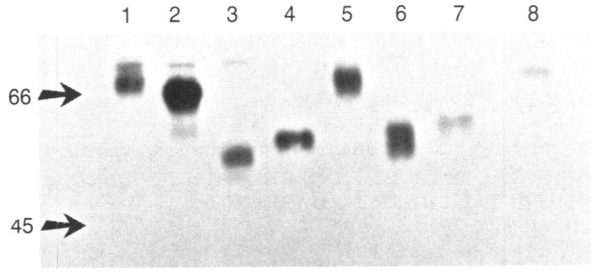

FIG. 5. Gel electrophoresis of proteins produced in CV1 cells infected with COOH-terminal anchorless recombinant virus. Pulse-chase was as in Fig. 3. Lane 1, complete HA; lane 2, cellular anchorless HA (H482); lane 3, cellular H482 and tunicamycin; lane 4, cellular H482 and endoglycosidase H; lane 5, N4OH482; lane 6, N4OH482 and tunicamycin; lane 7, N4OH482 and endoglycosidase H; lane 8, the secreted fraction of H482. Lane 8 was from a separate gel with a longer exposure.

clone (SH482, Fig. 4) for anchorless HA containing the native HA NH_2 terminus was also constructed and expressed.

As expected (5, 9, 27), anchorless HA (H482) was expressed intracellularly (Fig. 2*D*) but not on the cell surface (Fig. 2*H*). Immunoprecipitation of the medium bathing the SH482-infected cells yielded a detectable amount of secreted HA (Fig. 5, lane 8), indicating that the anchor function of HA had indeed been abolished. Secretion, however, was extremely slow, as reported previously by others (5, 9, 27). Virtually all of the intracellular H482 protein was endoglycosidase H sensitive (Fig. 5, lane 4), whereas the secreted protein was totally resistant (data not shown).

The chimeric HA (N4OH482) lacking the anchor at the COOH terminus was expressed as shown by immunofluorescence (Fig. 2*C*) and immunoprecipitation of ^{35}S-labeled proteins with anti-WSN antibodies (Fig. 5, lane 5). As with N4OH, cells infected with N4OH482 were negative for hemadsorption (not shown), and a small number of cells also exhibited positive surface immunofluorescence (Fig. 2*G*). Again, the amount of cell surface fluorescence as well as the number of positive cells were greatly reduced compared to cells expressing native HA. The glycosylation pattern of N4OH482 (Fig. 5, lane 4) was similar to that of N4OH (Fig. 5, lane 8)—i.e., very little, if any, of the carbohydrate present in N4OH482 was endoglycosidase H resistant.

We have clearly shown that the NH_2 terminus of NA can act as a signal by its ability to translocate signal-minus HA into the RER. Thus, both the signal function for translocation and the anchor function of NA are located at one end of the polypeptide spanning 35 amino acids. This region contains a cytoplasmic domain (6 amino acids) and a hydrophobic domain (29 amino acids). We have recently shown that the sequence conservation of the cytoplasmic domain is not critical in transport or in retaining enzymatic activity of NA (3). However, whether the cytoplasmic domain of NA that is highly conserved is important in directional transport in polarized cells remains to be seen. The hydrophobic domain of NA, which consists of 29 amino acids, is rather long compared to either the signal or anchor sequence of HA. It would therefore be conceivable that specific regions of this large hydrophobic domain are involved in signal and anchor functions either separately or cooperatively. Experiments to determine if the signal and anchor functions of NA can be dissociated from each other are necessary.

Clearly, the reduction in rate of transport of chimeric HA (N4OH) was not due solely to the "double anchor." The reason why these chimeras are blocked in transport is not clear; however, the experiments reported here suggest a few possibilities that can be further investigated. (*i*) Lack of cleavage of the signal sequence may interfere with the conformation as well as trimerization of HA, which may be required for transport. By specific modification of the cleavage site of the HA signal it should be possible to determine the role of cleavage in transport and structural assembly of HA. (*ii*) Since NA, which is normally not cleaved, can be transported from the RER via the Golgi apparatus into the plasma membrane, cleavage is not an absolute requirement for transport. Perhaps in chimeric HA, the NH_2 terminus of NA cannot cooperatively function with the rest of HA. Further investigation is necessary to distinguish between these and other possibilities.

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